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VOLUME 35, No. 3 JULY 1943

AN *IN VITRO* METHOD FOR THE CHEMOTHERAPEUTIC INVESTIGATION OF ANTHELMINTHIC POTENCY

BY ERNEST BALDWIN, *From the Biochemical Laboratory, Cambridge*

(With 11 Figures in the Text)

CONTENTS.—1. Introduction, p. 89. 2. Collection and maintenance of the material, p. 91. 3. Apparatus, p. 91. 4. Preparation and behaviour of the worm fragments, p. 92. 5. Preparation of drugs for testing, p. 95. 6. Discussion, p. 96. 7. Results and interpretations, p. 98. 8. Summary and conclusions, p. 110. Acknowledgements, p. 111. References, p. 111.

1. INTRODUCTION

The method to be described in this paper has been devised as part of a campaign designed to improve upon present-day drugs and methods for the treatment of animals infested with parasitic nematodes. The work was begun at the request of the Agricultural Research Council and has been financially supported by grants from the Council's funds.

An important part of the scheme consists in the preparation and chemotherapeutic investigation of new compounds likely to have anthelminthic potency, and it was necessary at the outset that a method should be available by means of which quantitative estimates of the efficiency of the new products could be quickly, if only rather approximately, obtained. 'The final test of the effectiveness of an anthelminthic', wrote Lamson & Brown (1936), 'is its administration to the host and the determination of the percentage of the parasites expelled.' But, as is well known, it is very difficult to find a convenient laboratory animal in which a controllable degree of nematode infestation can be maintained, and even when such a host can be obtained, the procedure of testing each of a large number of new compounds, the pharmacological properties of which are largely unknown, inevitably requires great expenditure of time, of money, and of the living material. There is therefore a strong case for the use of some *in vitro* method with the aid of which preliminary trials might be rapidly conducted on the new substances, from which the most promising could thus be selected for further investigation.

Much use has been made in the past of intact annelid worms, and of isolated muscle or nerve-muscle preparations from earthworms and leeches, as test materials for studies of anthelminthics, on the assumption, which has been widely made but would now appear to be quite unjustifiable, that drugs which exert an unfavourable influence upon annelid materials are likely to have a deleterious action upon nematodes also. It is, of course, well known that the neuro-muscular system of the Nematoda is morphologically unique, and we ourselves have found evidence to show that pig *Ascaris* is quite unaffected by a variety of drugs which are known to have a profound effect upon annelid worms. There can, in our opinion, be no doubt that the neuro-muscular mechanisms of the Nematoda, as represented by *Ascaris*, differ fundamentally from those of the Annelida, as represented by the leech and the earthworm. In any case, the use of annelid preparations has been strongly criticized by Lamson & Ward (1936), who reported that 'a comparative study of the lethality of 121 widely diversified chemical substances on both earthworms and pig *Ascaris* shows no correlation of action'. The more recent work of Oelkers & Rathje (1941) shows that in the few cases where *Ascaris* and certain annelid materials are affected by the same drug, there does exist some sort of parallel between their responses, but it seems to us that, even if this parallel is not purely fortuitous, it would be most unwise to rely upon it, especially when new and hitherto untried substances are to be examined for anthelminthic potency. For these reasons it seemed to us imperative to use some kind of nematode preparation as test material.

It is perhaps unfortunate that the term 'anthelminthic' has been applied without discrimination to drugs which have a noxious action upon parasitic worms which belong to more than one animal phylum. In general, it seems that if a given drug is active with respect to one parasitic nematode species it acts upon other species as well, and it is perhaps reasonable to expect that a drug which is lethal to one member of a given phylum will exert some sort of noxious action upon other members of the same phylum. But there is no justification for the supposition that it will act upon members of another phylum, even if these happen also to be parasitic. In point of fact it is of course known that, of the numerous drugs of which the anthelminthic properties are well established, some act specifically upon nematodes and others specifically

upon cestodes, while yet others attack members of both these groups. In the present paper we are exclusively concerned with the action of various drugs upon nematode worms, as represented by the roundworm of the pig, *A. lumbricoides*, and the use of the term 'anthelminthic' with reference to our own work must be taken to apply only to drugs acting upon members of this particular phylum.

The use of nematode material for *in vitro* experiments has been attempted or recommended by many workers. Thus von Schroeder (1885) reported the results of experiments in which intact parasitic worms were exposed to the action of various drugs. Trendelenberg (1916) carried out a few experiments upon isolated fragments of muscle prepared from *Ascaris*, but found the material so refractory that most of his classical experiments upon the pharmacology of santonin and its derivatives were in fact carried out upon earthworm preparations. Later, Rebello & Rico (1926) showed that tied-off pieces of *Ascaris* survive well and are capable of giving kymographic tracings; preparations of this kind have been extensively used in the study of anthelminthics by the Portuguese school (see da Costa, 1931, for example). This method seems however to have attained little popularity elsewhere, perhaps because no detailed description of the procedure appears to have been published.

Lamson & Brown (1936) have given a critical account of the earlier methods, and recommend a return to the use of intact specimens of pig *Ascaris*. These authors also investigated the possibility of using metabolic measurements of various kinds for the estimation of anthelminthic efficiency, but could find no measurable function suitable for this purpose. Dr M. C. A. Cross has carried out many experiments in this laboratory with the same end in view, but again without success. Lamson & Brown tested the kymographic technique adopted by Rico (1926), but rejected it as unsuitable for chemotherapeutic investigations. The procedure finally adopted by them was, in essence, the following: intact, healthy specimens of pig *Ascaris* are exposed under carefully standardized conditions to the action of the drug to be tested, after which they are washed and returned to a suitable medium for a definite period and then tested, again by a carefully standardized procedure, for viability. With the aid of this new method, Lamson *et al.* (1935–6) tested some hundreds of compounds for anthelminthic efficiency and brought to light the valuable nematocidal properties of hexylresorcinol.

While it can be discovered by this method whether a given compound does or does not have a lethal action upon *Ascaris*, the procedure gives little indication of the mode of action of the compounds tested. When, however, new substances are to be tested, it is desirable that the type of action produced by each member of a series should be distinguishable in order that attempts may then be made, by alteration of their chemical structure, to augment, suppress or otherwise modify some particular aspect of the activity. It was mainly for this reason that we decided to explore the possibilities of developing a kymographic technique, which might perhaps be expected to give indications of the mode of action of our compounds. Such a method would, it was felt, be more economical of living material than that of Lamson & Brown, for while in the latter method there is always a possibility that some of the test animals might have died even had they not been exposed to the action of any drug, a kymographic method of the kind we envisaged would involve a preliminary test of the activity of each preparation employed, so that its behaviour would be checked before application of the drug under examination. Unsuitable preparations could thus be rejected before the drug was applied, and there would thus be no need of the semi-statistical treatment involved in the method of Lamson & Brown.

In this country the choice of nematode material suitable for kymographic work is sharply limited. Few parasitic helminths can be kept alive in the laboratory for more than a day or two, and few are large or robust enough to do much mechanical work. In pig *Ascaris*, however, we have a form which fulfils these obvious requirements and is at the same time abundantly available. In a series of experiments carried out in this laboratory in collaboration with Dr Cross it was found, in confirmation of the results obtained by many other workers, that, on the average, healthy specimens of this worm can be kept 'alive', in the sense that they retain their motility, for 10–11 days, under quite simple laboratory conditions. Although the animals are undoubtedly in a state of extreme physiological decrepitude towards the end of this period, there is no reason to doubt that for the first 48 hr. at any rate they are for the most part in a physiological condition closely approximating to normal, and may be regarded as offering suitable material for our purposes. More is known about the general biology of this than of any other nematode species so that, although we lack much detailed information, it is at least true that we are not working against a background of complete ignorance and empiricism.

A. lumbricoides is, of course, only a single species, and it may be argued that results obtained in tests on this form will not necessarily be valid when we have to deal with other nematodes. It is known however (see Findlay, 1939, for example) that anthelminthics known to act upon species such as *Ascaris* do, in general, act also upon other

groups, such as hookworms, threadworms and some strongyles. Futhermore, we have submitted our *Ascaris* preparations to the action of a considerable number of drugs of which the anthelminthic potency with respect to nematodes is generally recognized, as well as to many compounds which possess no known anthelminthic efficiency. The results of these tests are described later in this paper, but for the moment it may be stated that the results point to a close correlation between positive experimental indications in our tests and anthelminthic activity in the drugs tested.

2. COLLECTION AND MAINTENANCE OF THE MATERIAL

Most workers have employed solutions containing *ca.* 1 % of sodium chloride as a medium for keeping *Ascaris* alive in the laboratory, a procedure which various workers have condemned (Lapage, 1937) on the ground that this medium bears no resemblance whatever to the normal environment of the worms. Some workers, e.g. Lamson & Brown (1936), have tried keeping *Ascaris* in more complex media such as those of Kronecker, Locke, Tyrode and Bunge, but found no increase of survival time. In our own work we have used a balanced saline medium throughout, and this we keep in the form of a concentrated stock of the following composition:

	g./l.		g./l.
NaCl	80	$CaCl_2$ anhydr.	2
KCl	2	$MgSO_4.7H_2O$	1

For use, one volume of this stock fluid receives one-tenth of its volume of 0·05 *M* phosphate buffer at *p*H 6·4 and is made up to ten volumes with distilled water. The product is referred to in what follows as Ringer's solution.

Our animals are collected at certain slaughterhouses in East Anglia, and in the ordinary way we have been able to obtain fresh supplies three or four times each week . A large thermos jar containing Ringer's solution at 40° C. is sent out from the laboratory. After a brief washing in warm water the worms are placed in the jar and sent by train to Cambridge. On arrival, when the temperature is usually 30 ± 1° C., the worms are washed if necessary, placed in large Petri dishes containing fresh medium, and kept in an incubator at 38–39° C. The animals are washed and the medium replaced every 24 hr., any worms that appear to be in poor condition being rejected.

If the worms are allowed to become cold, as has happened by accident on a few occasions, they seldom behave normally, and the experimental results obtained are untrustworthy.

3. APPARATUS

Our records are obtained with the aid of a light isotonic lever with a glass writing point. A time marker and a signal are also incorporated, and the levers of these are also furnished with glass writing points. The rate of travel of the drum can be varied to suit individual preparations, but usually lies between 2 and 5 mm./min. The recording lever gives a magnification of about 7 times, and a series of interchangeable weights allows the application of loads ranging from 0·25 to 1·5 g. For 'anterior' preparations the most suitable load is usually 0·5 *g.* while for 'intermediate' preparations 0·75 g. is usually satisfactory. These conditions are essentially the same as those recommended by Rico (1926).

The general lay-out of the apparatus is shown in Fig. 1. Our own apparatus is housed in a glass tank, the contents of which are stirred by a small electric motor and electrically maintained at 38–39° C. A constant water level is kept up by having a slow drip of water running in at *K*, the excess being run off by the siphon, *L*. The level can be adjusted by raising or lowering the vessel, *M*.

A stock of the Ringer's solution employed is kept under a constant head of pressure in a 15 l. bottle (*A*) and led off by siphon. A two-way tap (*B*) gives a choice between two routes (*C* and *D*), on one of which a short length of capillary tube is introduced (*C*). Two rates of flow of the medium are thus available: one of *ca.* 10 ml./min. is used for washing the worm fragments after mounting them for recording while the other, of *ca.* 70 ml./min., is used for washing out the test chamber (*G*) which accommodates the preparation. The medium passes through a glass heating coil (*E*) immersed in the thermostatic bath. A tap (*F*) is provided for stopping the flow of medium, which enters the test cell at the bottom, the overflow being sucked off from the top by a slight negative pressure controlled by the mercury bottle (*J*). The lower end of the worm preparation is attached to the glass hook (*H*) and the upper end to the recording lever. By turning the three-way tap (*I*) the vessel can be emptied from below. No provision for oxygenation of the medium is included or has been found necessary under our experimental conditions.

By using a small test chamber we are able to economize in drugs, but at the price of having the worm preparation sticking at the walls of the chamber when this is emptied. Occasionally this has been found to interfere with the behaviour of the preparation, which is then rejected, but in the vast majority of cases there seems to be no disadvantage except that the records are somewhat marred, but this can be overcome by lifting the tracing point while the cell is emptied and

refilled, an operation which can easily be accomplished within 30 sec. Only 10–15 ml. of the drug preparation are required.

4. PREPARATION AND BEHAVIOUR OF THE WORM FRAGMENTS

At first we made many attempts to obtain functionally active strips or rings isolated from the muscular body wall of our worms, but although motile fragments were occasionally obtained it soon became clear that preparations of this kind would be practically useless for routine purposes. Recourse was then had to tied-off tubular fragments of the kind proposed by Rico (1926), and these were found to be much more promising. After a large number of trials we developed the methods of preparation described below. The operations can be performed at room temperature, and are facilitated by the use of a glass plate backed by graph paper with cm. and mm. rulings.

Female worms only are used, and those of rather more than average size are preferred. Only those showing a good rosy colour and considerable activity are employed, and, in general, no worm is used that has been in the laboratory for more than 48 hr. Although we have sometimes obtained quite satisfactory fragments from worms after as much as a week in the laboratory, time spent in making such preparations is usually wasted. Two distinct preparations are normally set up from each worm: the selection and the mode of preparation of these fragments are somewhat arbitrary, but the procedure described here has been adopted, after many trials, as giving the largest proportion of useful preparations.

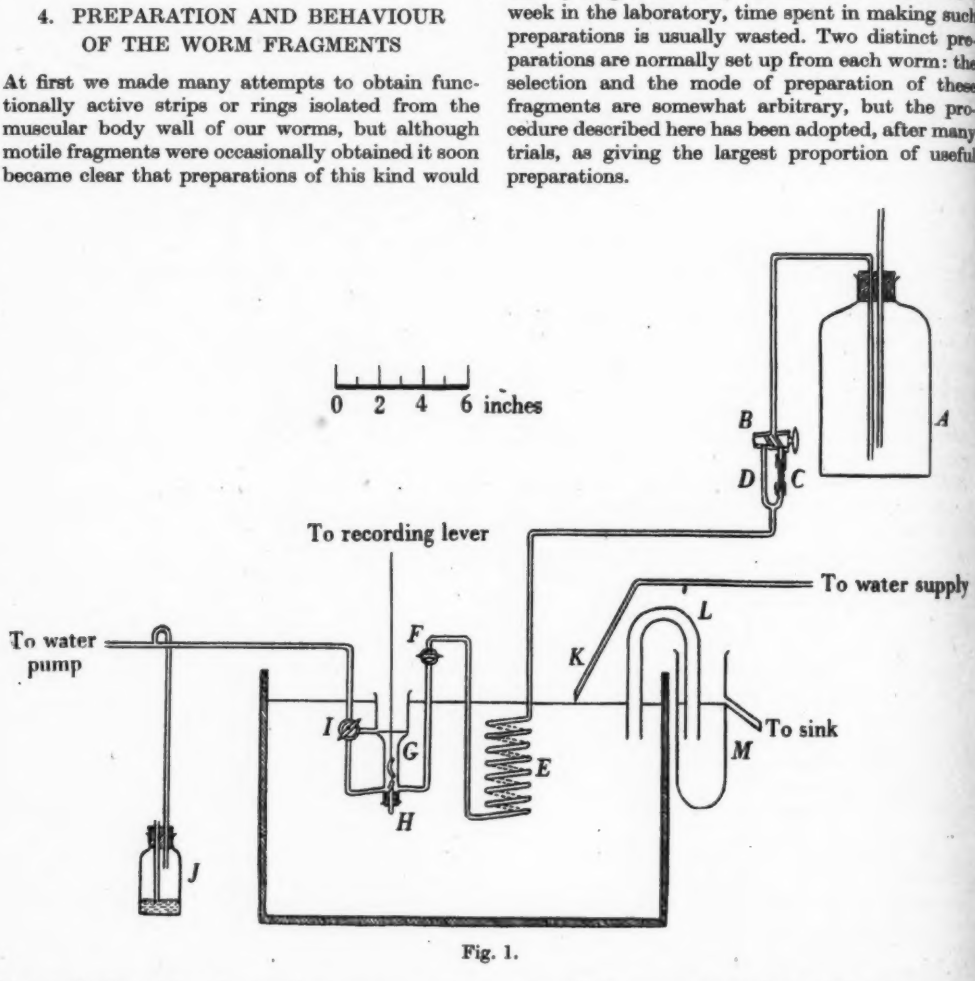

Fig. 1.

A suitable worm is selected, held horizontally, and dropped from a height of about 12 in. on to the bench. The whole body then contracts and the animal becomes and remains comparatively motionless long enough to allow the necessary operations to be carried out. If this preliminary treatment is omitted it frequently happens that the preparations contract considerably when the ligatures are applied and become too short to give satisfactory tracings.

The first or 'intermediate' preparation is made by tying a ligature with the finest grade of sewing silk at a position 2·8 cm. in front of the genital pore.

A second ligature is then applied about 3 mm. in front of the pore, and the remainder of the worm is cut away. This fragment corresponds to a ganglion-free nerve-muscle preparation, and contains nothing foreign to such a preparation apart from the intestine and, externally, the cuticle. The 'anterior' preparation is made by tying a ligature in or immediately behind the groove which lies at the base of the lips, i.e. rather less than 0·5 mm. behind the anterior extremity of the worm. Working against a black background it is not difficult to carry out this operation without the aid of a microscope. A second ligature is tied 2 cm. farther back and the rest of the animal is cut away. This preparation includes the muscular oesophagus, but it is unlikely that this contributes significantly to the movements of the preparation as a whole. What is more important is the fact that this fragment contains the so-called 'nerve ring', in which are collected the majority of the ganglion cells of the nervous system (see the description given by Rauther, 1925). This ring, which presumably discharges the functions of a primitive 'brain', lies about 1·5 mm. behind the anterior extremity of the worm.

By using both these preparations it should be possible to find out whether any given drug acts mainly or primarily upon the nerve ganglia or otherwise. Although with the majority of the compounds tested we have found that both regions respond in substantially the same manner, it is interesting to notice in passing that certain drugs cause paralysis of anterior preparations without affecting the intermediate fragments.

If the fragments are prepared in the manner indicated, and the ligatures are not tied so tightly as to cut into the tissues, about two-thirds of all the preparations settle down in time to give tracings of a rhythmic nature on a steady base-line. Our usual practice is to set up preparations from two or three worms at a time, each pair of fragments being set up in a boiling tube containing Ringer's solution and kept under observation in the thermostatic bath. Some of the anterior preparations develop a powerful activity which ceases shortly after a load has been applied to them, and for this reason we apply to each fragment a 'test load' (our own test loads are made from small glass beads) of about 0·5 g. and keep it under observation. In this way it is possible to select the useful preparations as they come into activity and to discard, without further waste of time, any which remain inert or behave abnormally. The time taken to recover from the operation is very variable, and as a rule we discard any preparations that have not become active after 60–90 min.

It is usually found that a preparation that has come into activity, and has remained active for 15–20 min., can be mounted with the appropriate load and will then continue, after a short interval during which it becomes adjusted to the new conditions, to move rhythmically on a steady base-line for several hours. A number of typical normal tracings are shown in Fig. 2. Sometimes the anterior preparations show short periods of quiescence as illustrated in Fig. 3*A* but such preparations are usually serviceable. Occasionally, however, anterior and intermediate preparations alike will go out of activity for many minutes (Fig. 3*B*). If this happens shortly after the administration of some drug, this effect will of course appear to be due to its action, and it is therefore necessary to repeat any experiment in which a positive result is obtained. Fortunately, however, this kind of behaviour is seldom encountered. In experiments in which the addition of a drug produces no change in the nature of the tracing, repetition is not strictly necessary if only qualitative information is sought. Nevertheless, we have normally repeated all our tests, and the results listed in the tables correspond in each case to experiments on at least two anterior and two intermediate preparations.

When quantitative information is required, the tests must be repeated several times since, while different individuals respond in substantially the same qualitative manner to a given drug, the time required for the production of a given effect varies considerably, probably because, as Dr A. R. Trim has found, the permeability of individual specimens varies rather widely.

Our present knowledge of the physiology of movement in *Ascaris* is too meagre to allow of any thoroughgoing analysis of the tracings furnished by our preparations, but useful supplementary information may be had by direct observation of the preparation itself. The movements of the tracing point do not correspond to simple longitudinal contractions and relaxations such as are observed with a simple strip of isolated muscle, and contractions of this kind are only seen under abnormal circumstances. The principal movements executed by our preparations consist of arcing in the dorso-ventral plane: as each arc is established the ends of the fragment are drawn more nearly together, so that the over-all length of the preparation decreases and an upward excursion of the tracing point is recorded on the drum.

In the intermediate fragments the pattern thus produced is a very simple, bold one, showing alternating up and down movements which correspond to, and are easily recognized as being due to, the dorsal and ventral arcs. Individual preparations vary widely with respect to the time scale (Fig. 2). No other major movements are to be seen in these preparations except in unusual cases. In the case of anterior fragments, however, the arcing is often accompanied by curious twisting movements, and

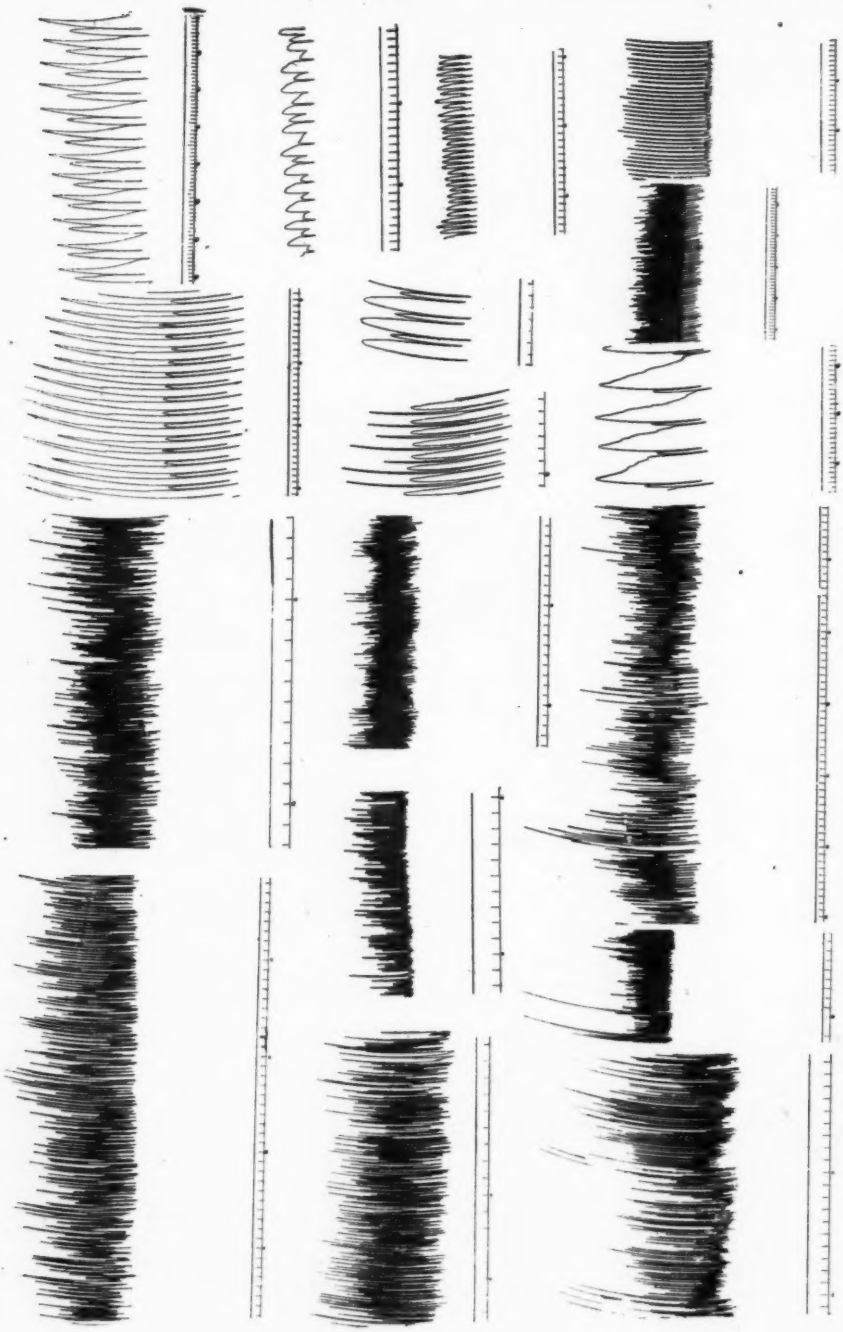

Fig. 2. Typical normal tracings: on the left are tracings of anterior and on the right of intermediate preparations.

it is usual to find that the arcs do not alternate strictly. The dorsal muscle mass, for example, may contract several times before the fragment turns over to arc one or more times in the opposite direction. The tracings obtained with anterior preparations are, in fact, always more complex than those with intermediate fragments, perhaps because the presence of the nerve ring confers upon the anterior region a greater versatility of movement (Fig. 2).

These tracings are liable to a variety of different modifications in response to different drugs. The rate and amplitude of the movements are usually affected, sometimes together and at other times

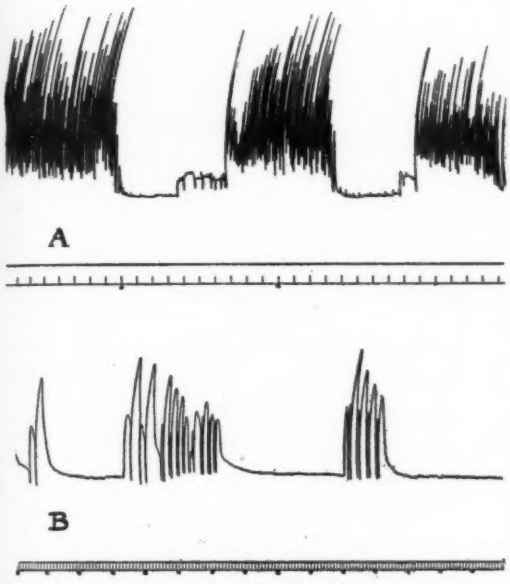

Fig. 3. Examples of 'resting' type of behaviour: A, anterior; B, intermediate.

independently, while the level of the base-line may rise or fall. Since the level of this line corresponds to the relaxed position of the fragment, we think that it may be interpreted as a measure of the average tone level of the preparation and when, in what follows, reference is made to changes of tone we mean that the level of the base-line of the tracings was correspondingly affected. As a general rule, both preparations react in a substantially similar manner to a given drug, and owing to the greater simplicity of the intermediate tracings we prefer to use these for critical testing, or when quantitative information is being sought, though this is not always possible.

5. PREPARATION OF DRUGS FOR TESTING

It is necessary in chemotherapeutic work of the kind with which we have been concerned to have some quantitative measure of the anthelminthic potency of each drug studied. This is not difficult when the compounds are soluble in water so that their effective concentrations can be accurately computed. But most useful anthelminthics are substances of relatively slight solubility: in every case these drugs are more or less toxic to the host, and for practical purposes relatively insoluble products are usually preferred on the presumption that they are less likely to be absorbed on a large scale by the host and so give rise to toxic symptoms. Lamson & Brown (1936) state that most useful compounds in this class have solubilities lying between *ca.* 1 : 1000 and 1 : 35,000, and it is also worthy of note that the melting points of most useful anthelminthics lie below about 80° C., as was first pointed out by Harwood (1934). This means that, in many cases, substances likely to be of practical value as anthelminthics can be exactly studied only in very dilute solutions and in this case long-period experiments would be necessary.

In their own method of *in vitro* testing, Lamson & Brown (1936) used each of the drugs to be tested in the proportion of one part to 1000 parts of diluent. The mixture was mechanically stirred at 38° C. for several hours before carrying out the actual test, and it is possible that the condition of a drug so prepared approximates fairly closely to that in which it would reach the parasites if administered to an infested host. But unfortunately the effective concentration of a drug put up in this manner is exactly known if, and only if, the compound dissolves completely. In general it might be supposed that the effective concentration would be equal to that in the watery phase, and that the particulate matter in suspension could be neglected. Nevertheless, in the course of several hours' vigorous mechanical agitation, a part or all of the undissolved material might pass into a state of colloidal dispersion, especially if, as is often the case, the drug is a liquid, and even, perhaps, if it is a solid of low melting-point. The possibility that drugs in an emulsified condition might be capable of penetrating into and acting upon nematode tissues seems not to have been investigated, although the use of solutions in ox bile has been adopted in many experiments by the Portuguese school, for instance by Rico (1926, 1927).

Working with a considerable number of com-

pounds of low melting-point we have found that even very sparingly soluble compounds can often be obtained in the form of emulsions containing 1 part of the drug in from 1000 to 5000 parts of the diluent, and that by taking advantage of the stabilizing properties of wetting agents such as sodium glycocholate, these emulsions can usually be made stable for periods of from one-half to several hours. On testing the activity of emulsions thus stabilized we found, rather contrary to expectation, that an emulsion containing, say, 1 : 1000 of a given drug is far more active than a suspension of finely divided crystalline material of the same concentration. Moreover, a 1 : 1000 emulsion is about twice as active as one at 1 : 2000 and so on, as judged by the time taken to produce a given response in our preparations. This effect is not due to the presence of the wetting agent as such, since it is without action when applied at the same concentration as is used for stabilizing the emulsion, nor does it make any appreciable difference to the activity of solutions of soluble compounds or to that of suspensions of particulate material to which it is added. These facts would seem to show that drugs applied in the form of finely dispersed particles of the colloidal order of size can pass through the nematode cuticle, though the mechanism of this penetration is still obscure. From the practical point of view this phenomenon is of great value since, by working with emulsions of this kind we are able to obtain rough quantitative data in experiments lasting only about half an hour, whereas periods running probably into many hours would be necessary if the drugs were only available in true solution.

There is at present nothing to justify the supposition that the effective concentration of a drug applied in the form of a 1 : 1000 emulsion is the same as would be that of a 1 : 1000 solution, so that it is not possible to compare with accuracy the activity of a water-soluble drug such as santonin with that of very insoluble materials such as oil of chenopodium or the halogenated hydrocarbons. But within a group of closely related chemical compounds of similar structure and properties, it is probable that the relative potencies of the individual members can be quantitatively assayed, if only approximately, in terms of the effects they produce when applied to the *Ascaris* preparations in the form of highly dispersed emulsions of comparable concentrations. For the chemotherapeutic investigation of groups of closely related compounds our method therefore possesses advantages which hitherto have not been available.

We have employed sodium glycocholate, which is a constituent of the bile of most vertebrates (Sobotka, 1937), for the stabilization of our emulsified drug preparations, usually at a concentration of 0·05 % and never more than 0·1 %. Dr A. R. Trim has found that the rate of penetration of the body wall of *Ascaris* by certain drugs is very much diminished in the presence of bile salt at concentrations of the order of 1 %, but at the concentrations at which we employ it, sodium glycocholate appears to have no appreciable effect. The compound has no action on our preparations at concentrations up to 0·1 % and possibly more. In some cases our drug preparations have also contained ethyl alcohol at 0·5 %, but our *Ascaris* fragments tolerate this reagent up to 1 % without showing any modification of their behaviour. Consequently we believe that the use of sodium glycocholate and of ethyl alcohol is justifiable at the concentrations actually employed.

Drugs are prepared for testing by solution in Ringer's solution in cases where they are sufficiently soluble. When this cannot be done but the compound has a melting-point below 100° C., the drug is suspended in the same medium with the addition of the appropriate quantity of sodium glycocholate. The mixture is heated on the water-bath to a temperature above the melting-point of the drug and is then vigorously shaken. Extremely violent agitation is often necessary, and must be maintained for several minutes at least. A sample of the freshly prepared emulsion is then set to take up the temperature of the bath, and used as soon as possible. For subsequent experiments the main bulk is reheated and again shaken since, even if the drug has not actually separated in the meantime, the emulsions sometimes decrease in activity on standing, probably on account of an increase in the mean size of the dispersed particles. Where crystallization takes place rapidly the stability of the emulsions can sometimes be increased by the inclusion of 0·5 % ethyl alcohol.

Very insoluble, high-melting compounds can often be obtained in emulsified form by dissolving the solid in absolute alcohol and adding the solution, with violent shaking, to hot Ringer's solution containing 0·05 % of sodium glycocholate. Preparations of this kind are best kept in the incubator, and should not contain more than 0·5 % of ethyl alcohol.

6. DISCUSSION

Our general procedure for routine testing is as follows. A number of worm preparations are set up, provided with test loads, and kept under observation. A fragment that has become active, and has remained active for at least 15 min. under its load, is mounted in the chamber, and washed for 2–5 min. A record is taken for at least 5 min. after the preparation has settled down to a steady level of activity. The recording lever is then lifted from the smoked surface, the vessel emptied from below by suction, and a sample of the drug preparation

to be tested, previously warmed by immersion in the bath, is carefully poured in from above. The lever is then reapplied to the drum and recording is continued.

Short period experiments are preferred since, although a good preparation will often remain active on a steady base-line for many hours, the amplitude and the general pattern of the tracings are liable to vary somewhat in the course of time, but these variations are seldom at all marked during the first 30–60 min. As a general rule it suffices to continue recording for only 30 min., and the majority of our experiments have been terminated after this period, since most of the drugs we have tested and found to be active produce paralysis of the preparations within 30 min. at concentrations of 1 : 1000 to 1 : 5000 when applied in a suitable form. This procedure has been in use with very satisfactory results in this laboratory for many months, but it is open to a number of objections on various grounds.

Any *in vitro* method based upon the use either of intact nematodes or of fragmentary preparations of nematodes suffers from certain inherent disadvantages and limitations. Methods of this kind are liable to detect anthelminthic potency with any drugs of a kind which, though they themselves are active, undergo inactivation when in contact with the tissues or digestive juices of the host. We have not so far encountered any such compounds, but it is known that certain synthetic organic arsenicals, for instance, are without action upon nematodes, but are converted into potent materials when administered to infested hosts (da Costa, 1931). The potential anthelminthic value of such compounds as these cannot, of course, be directly discovered by a method of this kind.

A special disadvantage of our own method is due to the fact that ours are essentially nerve-muscle preparations, and cannot therefore be expected to respond to any anthelminthics which act otherwise than on the neuro-muscular apparatus of nematodes. For tests of substances of this kind the use of the intact parasite, as in the method of Lamson & Brown (1936), may be expected to be more useful than ours. Most of the anthelminthics in common use at the present time do, however, act upon the neuro-muscular tissues, as is shown by the responses which they evoke from our preparations. The possibility that they act upon other tissues as well cannot, however, be excluded.

A particularly important criticism that may be made is that, since in our preparations the alimentary canal is completely occluded by the ligatures, drugs applied externally can only gain access to the tissues by traversing the cuticle, a structure which has the reputation of being very impermeable indeed. Its reputation for impermeability appears to derive mainly from the fact that most of the common histological stains pass only slowly through it (Lapage, 1937). But when active compounds such as santonin, hexylresorcinol, and even insoluble compounds such as oil of chenopodium in the form of emulsions, are applied to our preparations, their effects begin to be manifest within 2–3 min. as a rule, so that the inward passage of these compounds at least must take place very quickly indeed. Davey (1942) also finds that 'most of the successful anthelminthics...are capable of acting on nematodes through the cuticle'. Experiments carried out by Dr A. R. Trim have shown that certain drugs pass rapidly through the cuticle, and these facts, together with certain results obtained in Newcastle by Prof. Hobson and his coworkers, make it seem probable that the supposed extreme impermeability of the nematode cuticle is largely if not quite fictitious.

The fact that adrenaline, acetyl choline and a number of drugs related to them, even in concentrations far and away greater than those in which they act upon the neuro-muscular mechanisms of so many other organisms, fail to influence the behaviour of our *Ascaris* preparations, does indeed suggest that these substances may be unable to pass through the cuticle, and this possibility, if sustained, would of course constitute a very serious criticism of the method. It seemed to us most important, therefore, to find out whether these drugs produce any effect when applied by way of the gut. This was done by setting up preparations of the intermediate region with cannulae inserted into the gut and so arranged that the movements of the preparation could be recorded in the usual way, while a constant perfusion of the gut was maintained. The perfusion was carried out by means of a mechanically operated syringe of 50 ml. capacity delivering *ca.* 6 ml./hr. But in none of these experiments could we detect any significant change in the behaviour of the preparations as a result of the addition of adrenaline, pilocarpine, or acetyl choline with and without eserine, to the perfusion medium. Drugs such as santonin, thymol and hexylresorcinol, however, produced their usual effects, though they appear to penetrate more slowly when applied internally. Further details of these experiments will be published later.

As a general test of the applicability of the method, Dr G. Lapage kindly supplied to us seventeen preparations (A–Q), the chemical nature of which was not disclosed until later. Each of these was examined by the usual technique, and the substances were found to be sharply divisible into an active and an inert group on the basis of these tests. The drugs were applied, where necessary in emulsified form, at concentrations ranging from 1 : 1000 to 1 : 5000 and the results may be summarized as follows:

Active	Inert
A. Santonin	C. Ringer's solution
B. β-Naphthol	D. Sodium cetyl sulphate
E. Thymol	F. Neosalvarsan
H. Oil of chenopodium	G. Sodium glycocholate
K. Hexylresorcinol	I. Dextrin
L. Tetrachloroethylene	J. Sodium tauroglycocholate
N. Carbon tetrachloride	M. Gentian violet
O. Dichlorobutane	
P. Santonin	
Q. β-Naphthol	

It is clear from these results that the method was successful in detecting anthelminthic potency in all the compounds known to possess it, while negative results were obtained with the indifferent substances. To this there was one apparent exception, for cases of threadworm infestation can be successfully treated by oral administration of gentian violet (see, for example, Wright & Brady, 1940). However, as the mode of action of this dye is not at present known, and as our method has certain inherent limitations that have already been discussed, we did not feel that this single failure invalidates the application of our procedure to other compounds.

The results of this series of tests were interesting from other points of view. Thus it was evident, from the nature of the tracings, that A and P were identical or intimately related at the very least, and that the same was true of B and Q. Further, L, N and O produced very similar qualitative effects, suggesting that they must be closely related chemically and must probably act in much the same way. This particular indication was all the more striking since we had not at that time carried out any tests on any of the chlorinated aliphatic hydrocarbons. The three phenols, K (hexylresorcinol), E (thymol) and B, Q (β-naphthol), also showed evidence of structural relationships in that the final results of their action was similar, but so consistent were the results obtained with each drug individually that it was not difficult to distinguish between their effects in the earlier stages. Finally, a comparison of the tracings obtained in this group of experiments with the records of earlier tests, which had been made of all the active compounds apart from the chlorinated hydrocarbons, showed that the same general features, and many of the details, could be detected in practically every case when the effects of a given drug were examined. These facts offer convincing evidence of the specific nature of the responses of our preparations to drugs which are not very closely related, of the general similarity of the reactions provoked by more closely related compounds, and, above all, of the consistent nature of the effects recorded when any particular drug is employed.

In spite of the considerable measure of success and encouragement which came out of this series of experiments it is, of course, arguable that there may be many substances which would produce some kind of noxious effect upon our preparations while possessing no practical value as anthelminthics. Accordingly we have examined the reactions of the worm fragments to a larger number of drugs, some of known anthelminthic potency and established repute, and others with no established reputation for activity. The drugs of the latter group included a considerable number which, by analogy with their known effects upon other organisms, might have been expected to produce some sort of effect upon the behaviour of the nematode preparations. The results obtained are summarized in Tables 1–3, and leave no doubt that, except in a few cases, our procedure is capable of detecting anthelminthic potency in drugs which are known to possess it, while drugs with no known anthelminthic properties provoke little or no response in our preparations. These results may now be described in more detail.

7. RESULTS AND INTERPRETATIONS

Tables 1–3 summarize the results obtained in our experiments upon a total of fifty-eight compounds. All the tests were carried out by means of the procedure described above and in the majority of cases the experimental period was one of 30 min., though this was extended where it seemed necessary or instructive. The drugs were tested, either in solution or in the emulsified form at one or more of the following concentrations: 1:100,000, 1:50,000, 1 : 10,000, 1 : 5000, 1 : 2000, 1 : 1000, with higher concentrations in one or two cases. The results may conveniently be discussed under three headings, according as the drugs were without activity, influenced the behaviour of the anterior fragments mainly or entirely, or acted upon both preparations alike. It should be noticed that these experiments were carried out with a view to testing the general applicability of our method, and not with the intention of making an exhaustive study of the activities of the various substances used. Nevertheless a number of facts have emerged that seem to merit mention and brief discussion at this point although, in matters of detail, the observations reported here must be regarded as tentative.

Specimen records are reproduced in Figs. 4–11, and the following symbols are used. Each record is marked with the serial number of the worm employed, the intermediate preparations being denoted by an asterisk. Washing is indicated by the symbol $R \rightarrow$ and a stroke on the signal line, and cessation of washing by the symbol X. The operations of emptying and refilling the test chamber are marked on the signal line, thus, $\lfloor\!\rfloor$, and the time-marker intervals correspond to minutes in all the records.

Table 1. *Drugs without significant action on either preparation*

Compound	Highest conc. tested	Nature of preparation
Extractum filix mas	1 : 1000	Emulsion
Pelletierine tannate	Saturated	Solution
Pelletierine sulphate	1 : 1000	„
Gentian violet	1 : 2500	„
Phenothiazine	1 : 1000	Suspension
Caffeine	1 : 1000	Solution
Muscarine hydrochloride	*ca.* 1 : 1000	„
Atropine sulphate	1 : 1000	„
Cocaine hydrochloride	1 : 1000	„
Quinine sulphate	1 : 1000	„
Morphine hydrochloride	1 : 1000	„
Heroine hydrochloride	1 : 1000	„
Pilocarpine nitrate	1 : 1000	„
Pseudo-aconitine hydrobromide	1 : 1000	„
Eserine	1 : 5000	„
Strychnine nitrate	1 : 1000	„
Strophanthine	1 : 1000	„
Acetylcholine hydrochloride	1 : 5000	„
Choline chloride	1 : 1000	„
Adrenaline hydrochloride	1 : 5000	„
Ephedrine hydrochloride	1 : 1000	„
Benzedrine hydrochloride	1 : 1000	„
Tyramine hydrochloride	1 : 1000	„
Histamine acid phosphate	1 : 2000	„
Arcain sulphate	1 : 1000	„
Chloral hydrate	1 : 1000	„
Phenyl urea	1 : 1000	„
Ethyl alcohol	1 : 100	„
Acetarsone	1 : 500	„
Neosalvarsan	1 : 2000	„
Bayer 205	1 : 2000	„
Sulphanilamide	1 : 100	„
Sulphapyridine	1 : 1000	„
Sulphathiazole	1 : 2000	„
Umbelliferone	1 : 1000	„
Coramine	*ca.* 1 : 1000	„
Dextrin	1 : 1000	„
Sodium cetyl sulphate	1 : 2000	„
Sodium glycocholate	1 : 1000	„
Sodium tauroglycocholate	1 : 1000	„

(i) *Drugs without significant action* (Figs. 4–7)

Extractum filix mas (Fig. 6, 599 *) is a drug which enjoys a high reputation as an anthelminthic but its action is confined to tapeworms, and no effect was detected in our tests. Pelletierine likewise is a drug of which the action is specific for tapeworms and neither the sulphate (Fig. 7, 688 *) nor the relatively insoluble tannate, which is preferred in the treatment of tapeworm infestations, had any action upon our preparations. The fact that neither Extractum filix mas nor pelletierine showed any significant indication of activity serves to emphasize the fact, already mentioned, that our method can only be expected to give positive results in the case of anthelminthics which act upon nematodes; cestodes and trematodes, belonging as they do to a separate phylum, are liable to respond in a different manner and to different drugs.

Gentian violet, as has been pointed out, is an efficient drug for the expulsion of threadworms. Its mode of action, however, is not known and so the reason for the failure of our method in this case cannot be profitably discussed at present (see Fig. 4, 324). The case of phenothiazine is of greater moment. This drug has come into great prominence in recent years, especially in the veterinary field, where it is used extensively. We did not succeed in obtaining emulsified preparations of this very insoluble compound, and had to be content with 1 : 1000 suspensions made up from the 'Avlon dispersible powder' marketed by Imperial Chemical Industries, Ltd. and containing 90 % phenothiazine. This product readily yields a very fine, comparatively stable suspension when shaken with water. Our procedure gave uniformly negative results with this preparation, except that a slight depressant action upon anterior fragments was sometimes observed. The mode of action of phenothiazine is uncertain, but preliminary experiments carried out at Manchester by Dr A. G. Willis indicate that its now well-established lethal effect is due to a rather specific action upon the reproductive apparatus. If this should be confirmed, the failure of our method in this case will be explained: ours are essentially nerve-muscle preparations and cannot therefore respond to a compound which only acts on other parts of the organism.

For the study of compounds that act in this manner it is possible that some method, such as that of Lamson & Brown (1936), in which the intact parasite is used, would be more satisfactory than ours. It has been found by Dr G. Lapage and Prof. E. Friedmann (cf. Taylor & Sanderson, 1940) that saturated aqueous solutions of phenothiazine inhibit or suppress the development of eggs and larvae of mixed stomach worms taken from the sheep, and a separate method based upon the use of this material has been elaborated by these workers and will be published later.

Numerous alkaloids, including pelletierine, eserine, morphine, quinine and strychnine, have at one time or another been credited with nematocidal properties (see the table given by Lamson & Ward, 1932). According to our results, however, simple derivatives of these and a number of other alkaloids are devoid of action upon nematodes (see Fig. 4), and this, probably, is one reason why none of them has acquired any lasting reputation.

The complete failure of all the other drugs listed in Table 1 to influence the behaviour of our worm preparations is very striking. Many of these substances, especially the sympathetico- and the para-

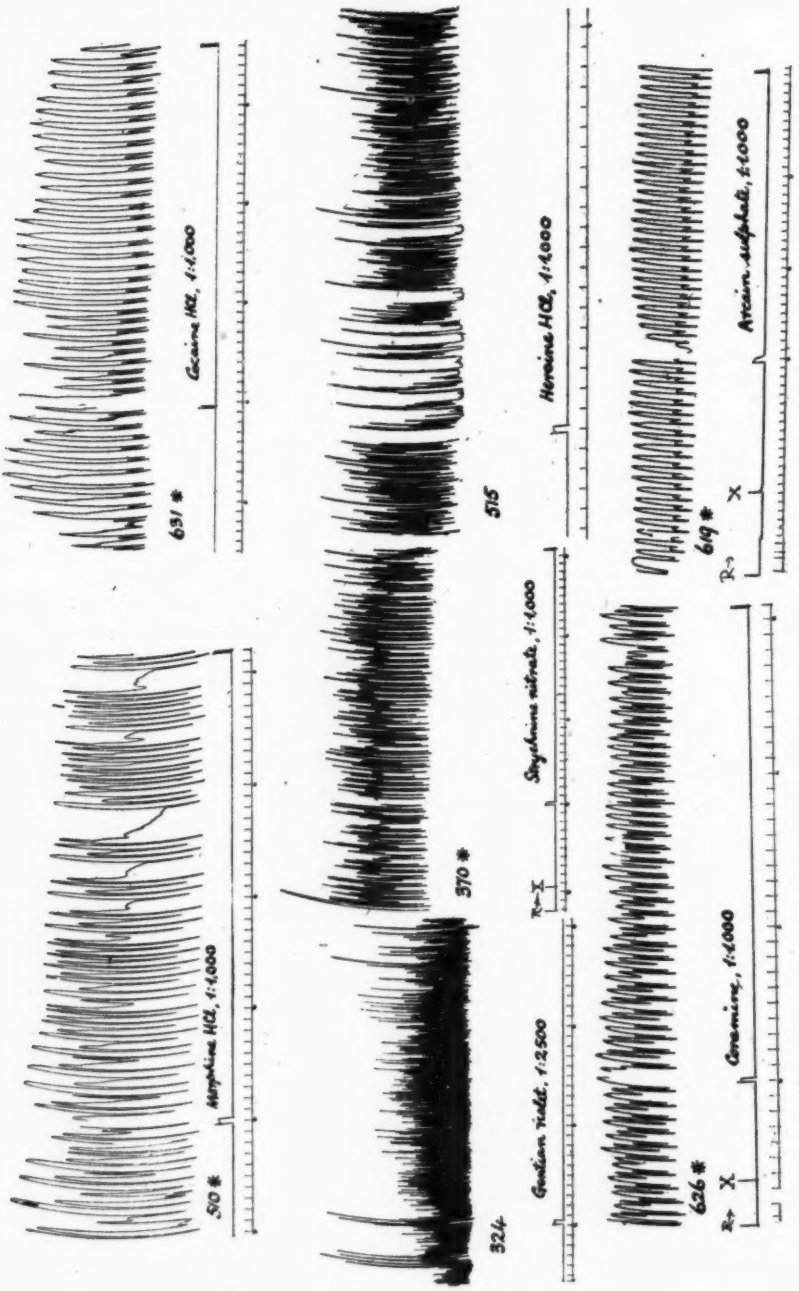

Fig. 4. Negative responses to various drugs.

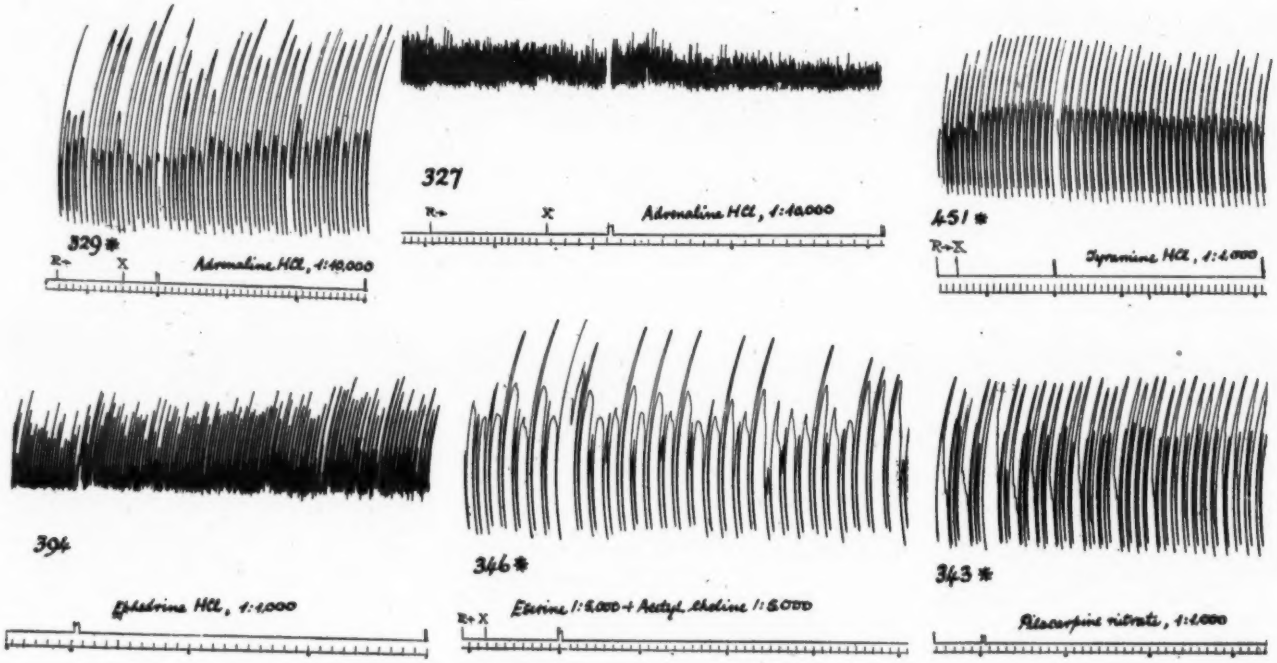

Fig. 5. Negative responses to various drugs.

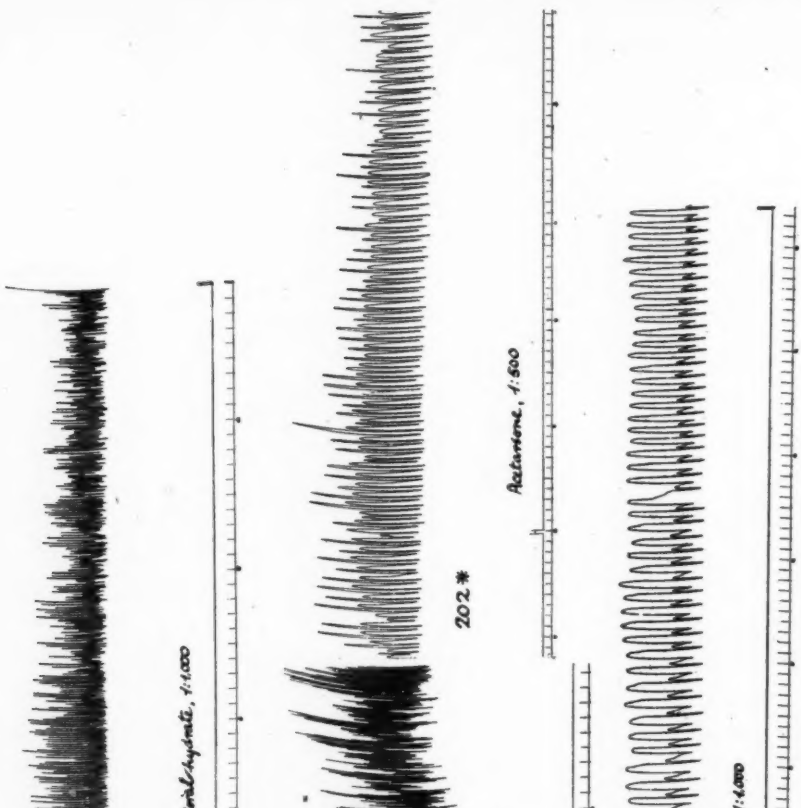

Fig. 6. Negative responses to various drugs.

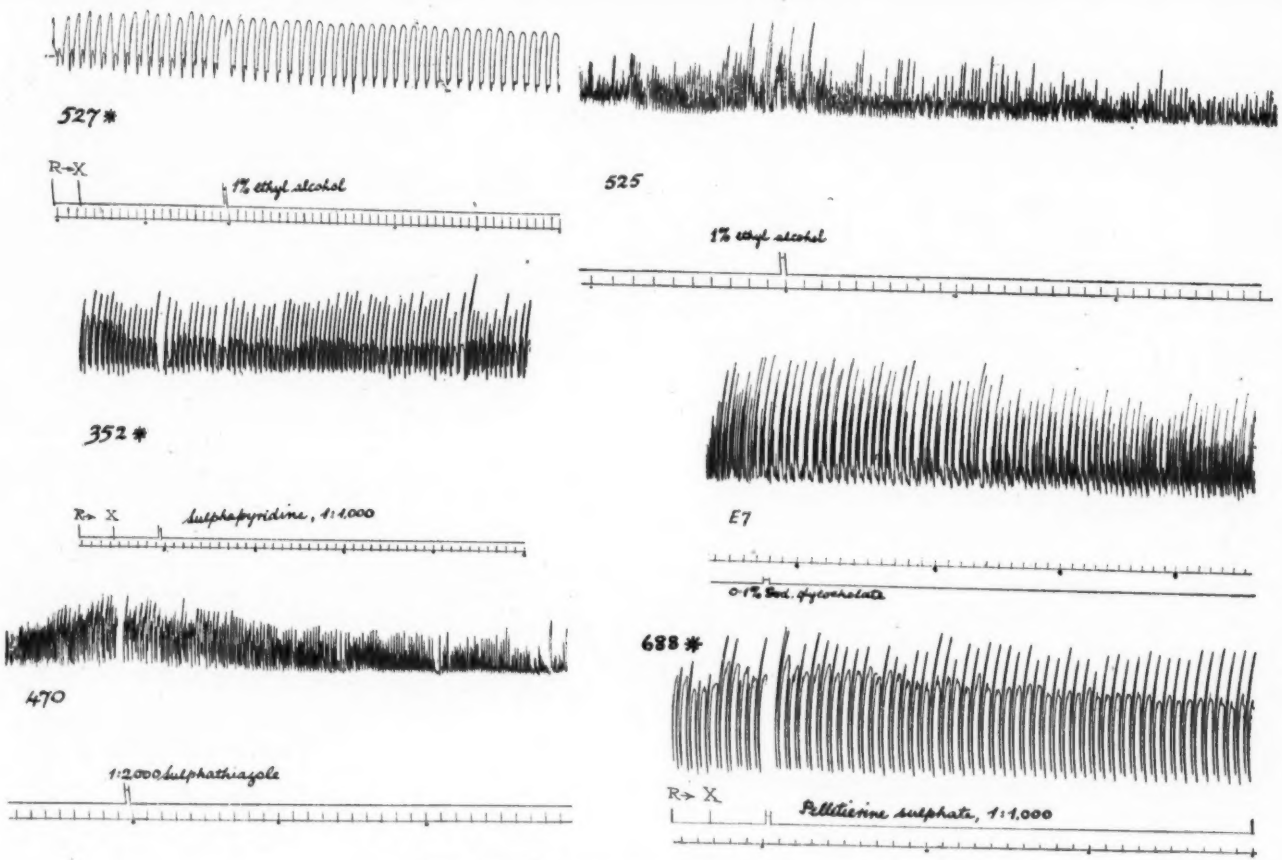

Fig. 7. Negative responses to various compounds.

sympatheticomimetic compounds, are known to act upon the neuro-muscular mechanisms of members of most animal phyla (cf. Fig. 5). Thus the musculature of the earthworm, for example, is profoundly influenced by such drugs as adrenaline, acetyl choline and pilocarpine, at exceedingly low concentrations, a fact which not only indicates how different must be the general organization of the neuro-muscular apparatus of the Annelida on the one hand and of the Nematoda on the other, but also emphasizes the unsuitability of annelid preparations as test materials for the study of anthelminthics acting upon nematodes.

The bacteriostatic sulphonamides (Fig. 7, 470, 352 *) have no activity. By contrast, acetarsone and neosalvarsan, though they themselves are inert (Fig. 6, 202 *), have been shown to be converted into powerful anthelminthic agents in the organism of the host, and da Costa (1931), to whom we owe this demonstration, speaks very strongly in favour of the use of acetarsone as an anthelminthic for administration to human patients infested with parasitic nematodes.

The inactivity of pilocarpine, strophanthine and umbelliferone is of interest in view of the fact that all three are lactones. Trendelenberg (1916) attributed the anthelminthic action of santonin to the lactone ring which its molecule contains, and believed that the action of pilocarpine and coumarine upon (earth)worm muscle is due to the fact that these compounds too are lactones. von Oettingen (1929) who, like Trendelenberg, relied upon the use of earthworms, prepared and studied a series of simpler lactones, three of which (β-angelica lactone, valerolactone carboxylic acid and the dilactone of acetone diacetic acid) proved to have *in vitro* a vermicidal activity as powerful as that of santonin. von Oettingen & Garcia (1929) then showed that the dilactone is a powerful anthelminthic, producing complete removal of *A. mystax* from infested cats in seven out of ten experiments. Other lactones possessing a high order of anthelminthic potency have been prepared by Gluschke (1932) and by Rosenmund & Schapiro (1934). While these results suggest that certain compounds may owe their anthelminthic efficiency to the presence of a lactone ring, the negative results which we obtained with pilocarpine, strophanthine and umbelliferone indicate that there is no specific relationship between the possession of the lactone structure on the one hand and that of anthelminthic potency on the other.

Finally, the fact that our preparations tolerate sodium glycocholate (Fig. 7, E 7) at concentrations of at least 0·1 % and are similarly unaffected by 1 % ethyl alcohol (Fig. 7, 525, 527 *) allows us to make use of these reagents in the preparation of drug emulsions.

(ii) *Drugs acting mainly or exclusively on anterior preparations* (Fig. 8)

All the compounds which we have tested and found to be active lead in the end to paralysis of our preparations, and we have found no drug that leads to more than transient stimulation of the anterior region. Often the fall of amplitude which leads to the paralysis takes place smoothly and without interruption, but it is at the same time not uncommon to observe short volleys of convulsive movements which appear after the normal movements have ceased, but which themselves disappear and give way to the final paralysis. Similar convulsive movements have also been observed in the intermediate preparations in cases where this region too is affected by the drug.

A number of drugs (Table 2) have been found to act only on the anterior region. The case of santonin is among these, and the mode of action of this drug is a problem that has been much discussed from time to time. Santonin, which is specific for nematodes among the parasitic helminths, is 'the chief anthelminthic frequently used in Great Britain' and 'the most reliable form of treatment for roundworms, and it can also be used for thread-worms when these cannot be removed by enemata' (Clark, 1940). Yet, as Findlay (1939) points out, it 'seems to have very little direct action on *Ascaris*'. Thus von Schroeder (1885) found that saturated solutions of santonin in dilute alkali had no lethal action upon roundworms in 40 hr. Trendelenberg (1916) attributed a powerful stimulating action to this drug which failed, however, in Lamson & Brown's (1936) experiments to kill any of the experimental worms in 3 hr. when applied in the form of a 1 : 1000 suspension in saline: oil of chenopodium applied in a similar manner killed 100 % of the worms in 30 min., and hexylresorcinol 100 % in 2 min. Oelkers & Rathje (1941) had similar experience, and came to the conclusion that the virtues of santonin have been overrated in the past. In our own experiments we found that santonin at 1 : 50,000 produces paralysis of the anterior fragments, with a marked fall of tone, in a few minutes (Fig. 8, 305). Some slight degree of activity often appears later, but this is never comparable with

Table 2. *Drugs acting mainly or exclusively upon anterior preparations*

Compound	Conc. producing paralysis in 20–30 min.	Nature of preparation
Santonin	1 : 50,000	Solution
Arecoline hydrobromide	1 : 1,000	,,
Coumarine	1 : 1,000	,,
Amytal	1 : 1,000	,,
Chlorbutol	1 : 1,000	,,

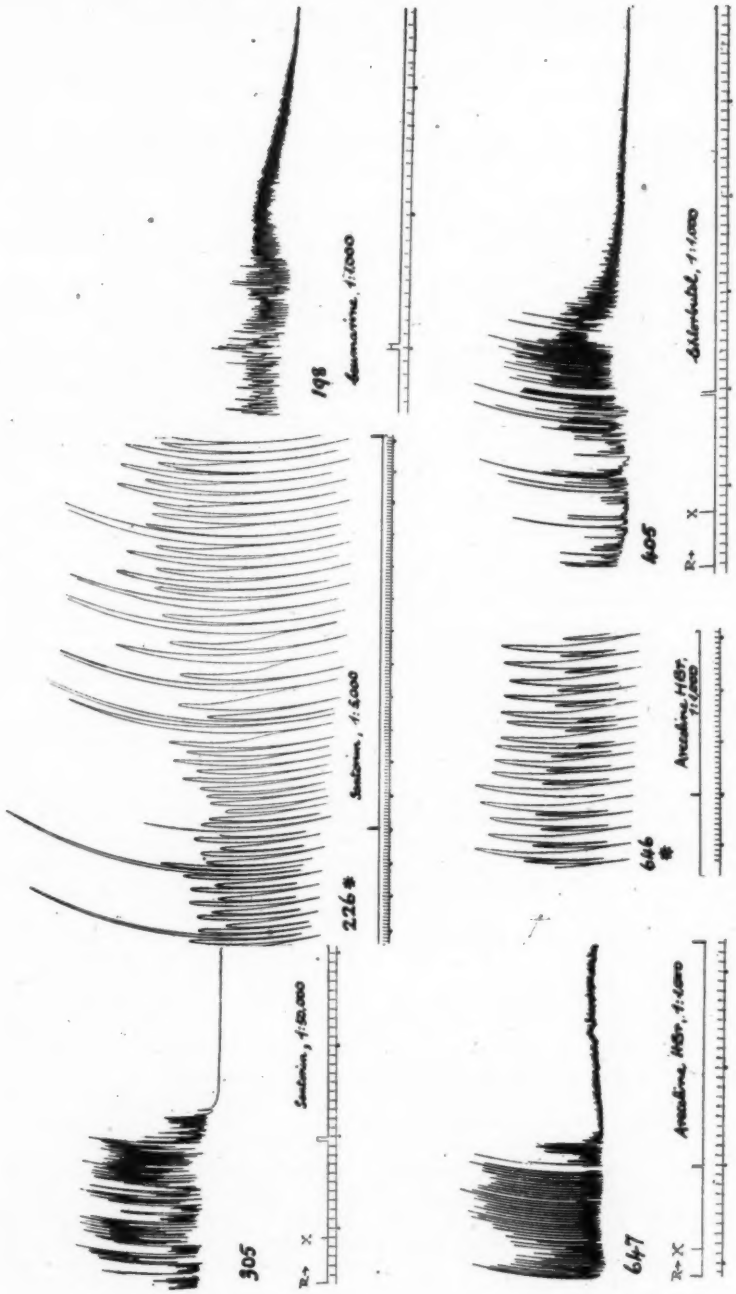

Fig. 8. Reactions to compounds acting mainly or entirely upon the anterior preparations.

that of the preparation before the drug was applied. No other compound we tested produced anything like this degree of effect at such low concentrations. At these low concentrations, the drug is without influence upon the intermediate preparation, but at 1 : 5000 it has a marked stimulant action. Its effect is not very obvious at first apart from the usual occurrence of an early fall of tone, but the amplitude of the movements gradually increases and continues to do so for a long time (Fig. 8, 226 *), though the normal pattern is usually maintained. These results suggest an explanation of the remarkable efficiency of santonin in spite of its seemingly slight action when applied to intact worms *in vitro*, for it is in the anterior extremity of the worm that the presumptive 'brain' is located. It seems likely, in view of our observations, that, while stimulating the hinder part of the worm to some extent, santonin at the same time deprives it of the coordinating impulses which may be supposed normally to originate in the nerve ring, as a result of which the animal is no longer able to maintain its normal position in the host, and can readily be expelled by giving a cathartic.

The other compounds in this group appear to act in a similar manner, except that no effect has been observed on intermediate preparations except in the case of santonin, while far greater concentrations of the other drugs are required to produce paralysis of anterior preparations. It is interesting to notice that the lactone coumarine (Fig. 8, 198) has this effect while the closely related substance umbelliferone, itself also a lactone, is inactive, and that chlorbutol (chloretone), which has been found (Hall & Wigdor, 1926) to have anthelminthic properties, is active in this respect (Fig. 8, 405), while chloral hydrate (Fig. 6, 484) is inert. The effects found with arecoline are interesting (Fig. 8, 647, 646 *). This drug shows some action upon roundworms (Hall & Wigdor, 1926), but owing to the far greater efficiency of santonin it is now only used against tapeworms, its action on these being, apparently, much more powerful.

(iii) *Drugs which act upon both preparations* (Figs. 9–11)

The remainder of the drugs we have tested act, usually in a fairly similar manner, upon the intermediate as well as upon the anterior preparations, and in every case the tendency is towards an eventual paralysis. Usually, however, the reactions are complex, and the onset of paralysis is often preceded by a greater or less degree of stimulation.

In order to obtain a basis for comparing the relative activities of a series of anthelminthic drugs in common use we have determined approximately the concentrations required to produce paralysis within 20–30 min., but it must be pointed out that since some of the compounds were tested in watery solution and others in the form of emulsions, no strict comparison between the two types can be justified. It is therefore convenient to consider the compounds in two groups, according as they are or are not water-soluble (Table 3).

Table 3. *Drugs acting upon both preparations*

Compound	Conc. producing paralysis in 20–30 min.	Nature of preparation
Hexylresorcinol	1 : 10,000–1 : 5000	Solution
p-Benzylphenylcarbamate	1 : 5000	Solution
β-Naphthol	1 : 5000–1 : 2000	Solution
Thymol	1 : 5000–1 : 2000	Solution
Nicotine	1 : 2000–1 : 1000	Solution
Phenylurethane	1 : 1000	Solution
Oil of chenopodium	1 : 5000	Emulsion
Carbon tetrachloride	1 : 2000–1 : 1000	Emulsion
Tetrachloroethylene	1 : 2000–1 : 1000	Emulsion
Dichlorobutane	1 : 1000	Emulsion
p-Cymene	> 1 : 1000	Emulsion
2-Methylnaphthalene	≫ 1 : 1000	Emulsion
Naphthalene	?	Emulsion

Considering first of all the compounds which are sufficiently soluble in water to be tested in solution, we find that hexylresorcinol is the most active member of the group, and is from 2 to 5 times more powerful than thymol and *β*-naphthol, which are of about equal potency. Lamson & Brown (1936) report that hexylresorcinol killed 100% of their experimental worms during 2 min. exposure, thymol requiring 30 min. to produce the same effect. Lamson & Ward (1932) were of opinion that hexylresorcinol acts primarily upon the nematode cuticle, since worms that have been killed by means of this drug show cuticular blistering. Our observations, however, show that the worm fragments are completely paralysed long before any blistering can be detected, and we cannot agree that hexylresorcinol owes its powerful nematocidal efficiency to its action upon the cuticle.

All three of the phenols studied act similarly in that they produce eventual paralysis of both preparations (Fig. 9), the paralysis being succeeded by contracture of the muscles. This phenomenon is not usually seen unless the drugs are applied at rather high concentrations (1 : 2000–1 : 1000) or are allowed to act for an hour or more. The contracture sets in most rapidly in the case of thymol, less in the case of *β*-naphthol, and still less in that of hexylresorcinol. The phenomenon is well illustrated for the case of thymol in Fig. 9 (237 *). In many cases it seems that the muscle mass of one side is affected before that of the other, and in the instance just mentioned the preparation, after a period of comparative quiescence, slowly executed a powerful

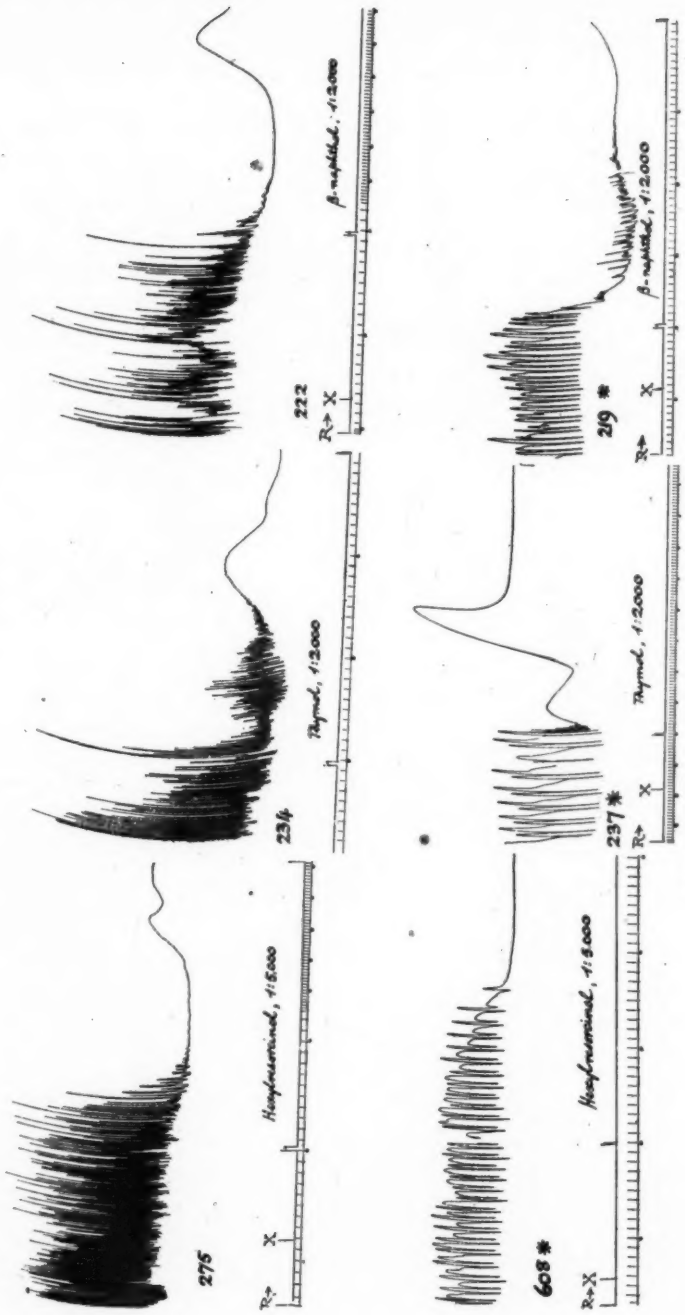

Fig. 9. Responses to some phenolic anthelmintics.

Fig. 10. Responses to various anthelminthics.

arc due to the contracture of the musculature of one side, and this arc slowly passed off as the other muscle mass went into contracture. The preparation then remained practically straight but markedly shorter than before, and this over-all shortening of the preparation is shown by an elevation of the base line. Similar effects are seen with anterior preparations, but in these the longitudinal contraction resulting from contracture is less evident and the base line rises correspondingly less. To this extent the responses provoked by these three phenols are similar.

Specific differences between the phenols are noticeable before paralysis supervenes. Hexylresorcinol and β-naphthol (Fig. 9, 275, 222) both produce a rapid fall of amplitude and tone level in the anterior fragments and this leads on to paralysis and later to contracture. In the case of thymol, however, the fall of amplitude is characteristically followed by a secondary rise and a further fall of amplitude (Fig. 9, 234), leading on to contracture in such a way that the period of quiescent paralysis is not observed in this case. When the drugs are allowed to act upon intermediate preparations, hexylresorcinol produces a fall of amplitude followed by paralysis (Fig. 9, 608 *) and then, after some time, by contracture. Thymol causes at first a marked acceleration of the movements, which rapidly fall off in amplitude to be followed by contracture without any intervening period of complete quiescence (Fig. 9, 237 *), while β-naphthol (Fig. 9, 219 *) produces on the intermediate fragments an effect which to some extent resembles that of thymol on the anterior region (Fig. 9, 234). These individual differences have repeatedly been observed.

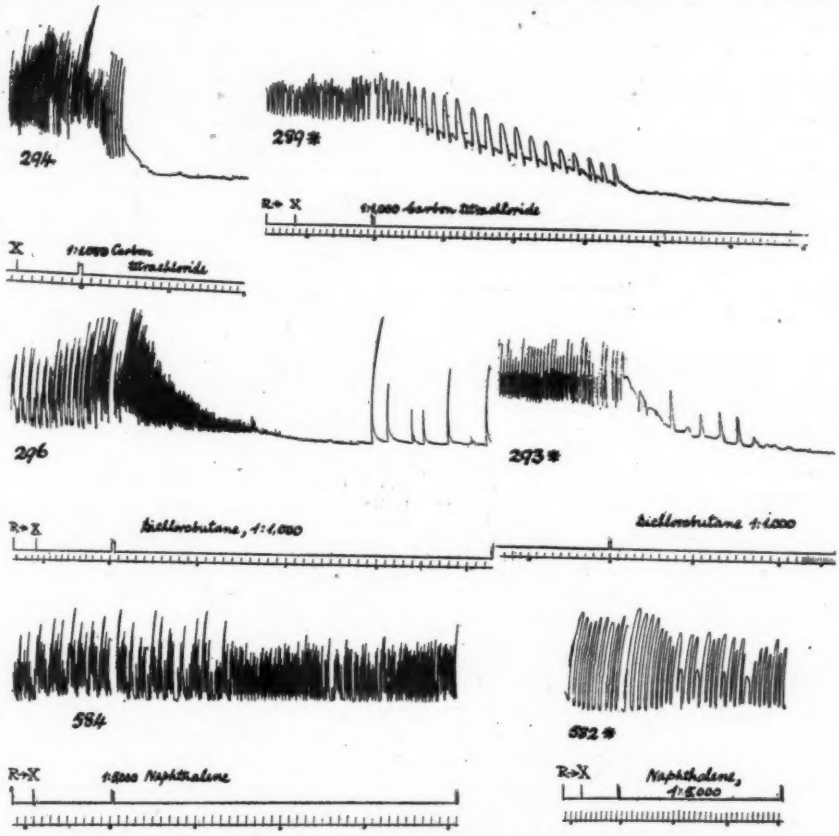

Fig. 11. Responses to some hydrocarbons and chlorinated hydrocarbons.

p-Benzylphenyl carbamate, the active constituent of 'Butolan', appears to be rather less active than hexylresorcinol and produces on anterior preparations a gradual fall of amplitude without much

change either of frequency or of tone (Fig. 10, 582). The movements of the intermediate region are rather differently affected: here the amplitude again falls but the frequency of the arcs is much increased and there is usually a slight fall of tone (Fig. 10, 587 *). Phenylurethane, so far as we know, has not been used as an anthelminthic. Its action resembles but is about five times weaker than that of *p*-benzylphenyl carbamate, and it may be that the two compounds, both of which are related to urethane, act similarly. Phenyl urea (Fig. 6, 464) is inert.

The action of nicotine is illustrated in Fig. 10 (J 19, 168 *) and calls for little comment, though the resistance of our preparations to this drug is certainly striking.

Of the drugs tested in emulsified form, oil of chenopodium was the most active. This preparation contains 60–77 % of ascaridole and about 22 % of *p*-cymene according to Oelkers (1940), who states that *p*-cymene is about as toxic as ascaridole itself towards *A. megalocephala* (*Parascaris equorum*). In the later paper of Oelkers & Rathje (1941) *p*-cymene was reported to be much less toxic than ascaridole towards *A. lumbricoides*, and our own observations indicate that towards this latter species it has about one-fifth of the activity of oil of chenopodium, so that the activity of the latter upon our preparations must be due, in the main, to its content of ascaridole.

Oil of chenopodium produces diminution in the amplitude of both fragments (Fig. 10, 255, 253 *) with little or no change in frequency; the tone of anterior preparations is not much affected while that of intermediate fragments falls markedly. The effect of *p*-cymene (Fig. 10, 588 *), though much weaker, is qualitatively not unlike that of oil of chenopodium.

Lamson & Brown (1936) state that 100 % of their experimental worms were killed in 30 min. by oil of chenopodium and 100 % by tetrachloroethylene in 2 hr., while carbon tetrachloride was fatal to only 10 % in 3 hr. In our experiments, however, tetrachloroethylene and carbon tetrachloride were about equally potent and only 2–5 times weaker than oil of chenopodium. The third member of the series of chlorinated hydrocarbons, dichlorobutane, was rather less active than the other two. All three compounds produced paralysis with a fall of tone, the latter being more marked in intermediate than in anterior preparations (Fig. 11, 289 *, 293 *). Although the effects of these drugs bear a superficial resemblance to that of oil of chenopodium there is evidence that they include an element of irritation. In anterior preparations the fall of tone is preceded by a short but well-marked rise (Fig. 11, 294, 296), while short volleys of convulsive arcs often appear before the intervention of complete paralysis. These are well illustrated for the case of dichlorobutane (Fig. 11, 296). Similar convulsions were sometimes observed in intermediate preparations also.

We also carried out some experiments upon naphthalene, but failed to obtain stable preparations more concentrated than 1 : 5000. At this concentration the drug seems to be almost devoid of activity (Fig. 11, 584, 582 *) although it was at one time widely used as an anthelminthic. We also tested 2-methylnaphthalene which, unlike the parent hydrocarbon, is liquid at the temperature of our experiments. In this case we were easily able to have emulsions at 1 : 1000, but even at this concentration only a feeble action was detectable. Rico (1927), working with saturated solutions of these two compounds in 2 % ox-bile, reported that both drugs produce a remarkable rise of tone, the activity then gradually falling off and giving way after 2 hr. or more to paralysis. In view of the disparity of our respective results it is tempting to suggest that Rico's results and also, perhaps, the one-time reputation of naphthalene as an anthelminthic, may have been due to the presence of traces of highly active impurities such, perhaps, as β-naphthol, in the drugs employed. In any case the study of naphthalene and its derivatives would probably repay further investigation.

It will be seen that, in the main, the results of our tests lead us to arrange the drugs which we have examined in an order of potency which agrees for the most part with that found by Lamson & Brown (1936). Our records, which are very consistent for each of the drugs tested, show that not all anthelminthic drugs act in the same manner, and even if we are at present too ignorant of nematode physiology to offer any useful or convincing explanation of the effects we observe, it does seem likely that pharmacological experiments along lines similar to those of our own experiments may well be of value in the elucidation of the at present obscure problems of the neuro-muscular physiology of this peculiar group of animals.

8. SUMMARY AND CONCLUSIONS

A method is described for the detection of anthelminthic potency. The procedure does not call for exceptional technical skill, is relatively economical of drugs and living material alike, and is rapid and convenient in application. It has certain limitations such as are inherent in any *in vitro* method, and a special limitation in that it is not applicable to the study of drugs which act otherwise than on the neuro-muscular mechanisms of nematodes. Within these limitations it responds to compounds of known anthelminthic efficiency and shows little or no response to compounds known to be devoid of anthelminthic value. Approximate quantitative data can be obtained with its aid, the reactions provoked by

a given drug are substantially the same in different individuals, and each test provides a visible record of the influence of the drug under examination.

We do not claim that this procedure can in any sense replace experiments upon infested hosts, but must rather be followed up by such experiments when a promising degree of anthelminthic potency has been detected with its aid. We do, however, believe that it has a very real usefulness within the field to which its application is limited. As a preliminary device which allows the selection of promising compounds from large groups of new synthetic products it has already demonstrated its value in tests carried out in this laboratory on a series of about fifty new compounds prepared by Prof. Friedmann.

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SOME NEMATODE PARASITES OF EARTHWORMS FROM THE INDO-MALAY REGION

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(With 38 Figures in the Text)

Some account is given in this paper of certain nematodes obtained by Prof. G. E. Gates from the coelomic cavities of various species of earthworms from South India, Assam and Singapore. The material was kindly submitted to the writer by Prof. Gates in 1936, 1937 and 1939. Of the forms included in it several were represented by specimens insufficient or unsatisfactory for adequate description, and it has seemed advisable to deal only with those species of which a reasonably full account can be given. Of these there are seven, all of which are thought to be new. Four of them can be assigned to a known genus, *Synoecnema* de Magalhães, 1905; the other three probably represent as many new genera.

Related forms. The adult nematode fauna of the body-cavities of Oligochaeta is probably quite extensive, but has so far been little explored. Species are known from Europe, the Mediterranean region, Africa, Ceylon, Burma, Annam, New Guinea, and North and South America. It is probable, therefore, that earthworms all over the world harbour such parasites. Most of the species known to occur in this highly specialized habitat are very minute, and their morphology is not easily studied. The only publication dealing with them at all comprehensively is that of Pierantoni (1916), who recognized among the forms studied by him three families, which he named Cephalonemidae, Onycidae and Drilonemidae.

The family name Cephalonemidae (*sensu* Pierantoni) is inadmissible, since the generic name *Cephalonema* Pierantoni cannot stand, being preoccupied by *Cephalonema* Stimpson, 1857 (nemertine) and *Cephalonema* Cobb, 1893 (nematode). The family name Onycidae (in Pierantoni's sense) is also inadmissible, since it is not formed from the name of a genus contained in the family, and has nothing to do with the genus *Onyx* Cobb, 1891. The only genus placed by Pierantoni in this family is *Dionyx* Perrier, 1881, the name of which is also preoccupied. Baylis & Daubney (1926) briefly discussed Pierantoni's classification, and expressed the view that all the genera recognized by him (which they reduced to five) could be assigned to a single family, for which they adopted the name Drilonemidae Pierantoni. This name has been justifiably emended to Drilonematidae by Chitwood (1935). Baylis & Daubney left open the question of the position of this family in the general scheme of classification of the Nematoda. Wülker (1926) regarded it (or at least the genus *Dicelis*) as probably closely related to the Anguillulinidae [=Tylenchidae]. He apparently based this view chiefly on a resemblance between the condition of the female genital organs in *Dicelis* and that in *Tylencholaimus stecki* Steiner. But Chitwood & Chitwood (1937), in their classification, separate *Tylencholaimus* widely from the 'Tylenchidae'. They place the subfamily Tylencholaiminae in the order Enoplida, superfamily Dorylaimoidea, whereas the 'Tylenchidae', according to them, belong to the order Rhabditida, superfamily Tylenchoidea. Not only so, but these two orders are assigned by Chitwood & Chitwood to different subclasses, the Rhabditida being placed in their subclass Phasmidia and the Enoplida in the Aphasmidia. The family Drilonematidae, however, is accommodated by these authors in an appendix to the superfamily Rhabditoidea, so that it occupies a position between that superfamily and the 'Tylenchoidea'. They thus seem to agree in principle with Wülker with regard to its probable affinities. They do not mention Pierantoni's other two families, and presumably accept the suppression of them.

The genus *Dionyx* Perrier was treated by Baylis & Daubney (1926) as a synonym of *Synoecnema*. In the light of the present study it seems probable that this step was unjustified. In all the species of '*Dionyx*' of which the male is known, there are two spicules and one or two accessory pieces. In the genotype of *Synoecnema*, *S. fragile* de Magalhães, 1905, no such structures have been described. Spicular structures seem also to be entirely lacking in the first four species to be described below. These all evidently belong to the same genus, and, as they have this character in common with *S. fragile*, they are assigned to *Synoecnema*. It seems reasonable to regard the related species in which spicules are present as belonging to a distinct genus. The name *Dionyx* cannot be revived for this, since it is preoccupied by *Dionyx* Le Peletier and Serville, 1825 (Coleoptera). The question arises whether *Ungella secta* Cobb, 1928, is congeneric with the species assigned by Perrier and by Pierantoni to *Dionyx*. If it were so, the name *Ungella* would be available

to replace '*Dionyx*'. *Ungella secta* is described in much greater detail than are any of the species of '*Dionyx*'. In general it bears a very strong resemblance to them, but it has one feature which, if present, has not been noted in them, viz. the presence of paired subventral caudal alae (described by Cobb as a 'bursa') in the male, and of several pairs of papillae with long pulps ('whiplash-like ribs') traversing these alae. The hooks with which the mouth is armed also appear to be more highly developed, forming a definite, protrusible 'onchium', or fused pair of 'onchia', and the oesophagus shows some indication of a median swelling in addition to the posterior swelling which alone is described for '*Dionyx*'. The oesophagus is, in fact, rather of the form that seems to be characteristic of *Synoecnema*. There is a pair of well-developed lateral 'suckers' on the tail in both sexes of *Ungella*, and these structures have not been described for '*Dionyx*', though they may have escaped detection. The same applies to the special cervical gland, opening by a dorsal pore on the head, described by Cobb for *Ungella*.

These differences suggest that it may be wise to allow *Ungella* to retain its independent generic status for the present, and it is therefore necessary to rename *Dionyx* Perrier. The name **Onychonema** is proposed, and the genus will contain the following species: *Onychonema lacazii* (Perrier, 1881) (genotype), *O. cognettii* (Pierantoni, 1916), *O. minutum* (Pierantoni, 1916), *O. acutifrons* (Pierantoni, 1916) and *O. guineense* (Pierantoni, 1916). *Synoecnema fragile* de Magalhães, 1905, will revert to its original status as the genotype of an independent genus, *Synoecnema*, with *Dionyx fragilis* Pierantoni, 1916, as a synonym. *Ungella secta* Cobb, 1928, remains the type and at present sole species of *Ungella*.

The present collection throws no fresh light on the new genera proposed by Pierantoni (1916, 1923). Chitwood (1935) has designated *Mesonema acuminatum* Pierantoni as type of *Mesonema* Pierantoni, 1916, and *Opistonema minutum* Pierantoni as type of *Opistonema* Pierantoni, 1916. He does not state whether he considers these two genera valid. The writer is still of the opinion expressed by Baylis & Daubney (1926) that the grounds on which they were separated from *Drilonema* by Pierantoni were inadequate. *Cephalonema* Pierantoni, 1916, was renamed *Pierantonia* by Baylis & Daubney (1926). Wülker (1926) has amplified the description of *Dicelis filaria* Dujardin, 1845, the genotype of *Dicelis*, to which Beddard (1883) added the species *D. pleurochaetae*, and Chitwood & Lucker (1934) have described a further species, *D. nira*. These descriptions confirm the validity of the genus *Dicelis*, but do not appear to necessitate any change in its position as a member of the family Drilonematidae.

Methods. The nature and condition of most of the present material rendered its adequate study very difficult. The nematodes had been obtained during the dissection of preserved specimens of the hosts, so that they were seldom in a very well-preserved state, and were always extremely fragile. Attempts were made at first to study them after simple clearing in lactophenol and temporary mounting in this medium or in glycerine, under a supported coverglass. This method, however, proved useless for observing many of the details of structure. Better results were obtained with specimens stained with Mayer's paracarmine and mounted in Canada balsam. Before staining it was generally found an advantage to soak the specimens for some hours or days in lactophenol. This counteracted any shrinkage that had occurred in their preservation. Acid alcohol was used to remove the lactophenol, and this at the same time tended to remove any discoloration resulting from the specimens having been kept for some years in corked tubes. The specimens were then passed through 70% alcohol into the stain, which was used at full strength. After staining for a few minutes and differentiating for a few seconds only in acid alcohol, they were again brought into 70% and thence into 80% alcohol in a solid watch-glass. Creosote was then added drop by drop round the edges with a pipette, and allowed to run under the alcohol. As the specimens sank into the creosote, the supernatant alcohol was pipetted off and fresh creosote added. After soaking in pure creosote until thoroughly dehydrated and cleared, the worms were mounted either in balsam dissolved in creosote or in a mixture of xylol-balsam and creosote. By the use of this method shrinkage was very largely avoided and, as much of the structure of the worms had to be studied under a $\frac{1}{12}$ in. objective, it was an advantage to have them mounted in a medium that would become solid. The chief disadvantage was the relatively high refractive index of the mounting medium, which rendered good definition of cuticular structures difficult to obtain. In making out the minute details of these very small worms an apochromatic oil-immersion objective and compensating oculars were found of great assistance.

Family DRILONEMATIDAE

Genus **Synoecnema** de Magalhães, 1905

Emended diagnosis. Mouth subterminal, turned dorsally and overhung by two recurved hooks springing from its anterior (ventral) margin. Oesophagus slender, with a posterior bulb and sometimes with a slight median swelling. Tail of male relatively long and usually tapering; sometimes with a pair of lateral pits or 'suckers'. Spicules and accessory piece apparently absent. Vulva either in

front of or behind the middle of the body. Female genital tube single, anterior, reflexed. Sexes often permanently *in copula*.

Hab. Free in the coelomic cavities of earthworms.
Genotype. S. fragile de Magalhães, 1905.

Synoecnema hoplochaetellae, n.sp.

(Figs. 1–7.)

Host. Hoplochaetella anomala Stephenson, 1920 (coelomic cavities of anterior segments).

Locality. Mysore, south bank of Sharavati river (Jog falls).

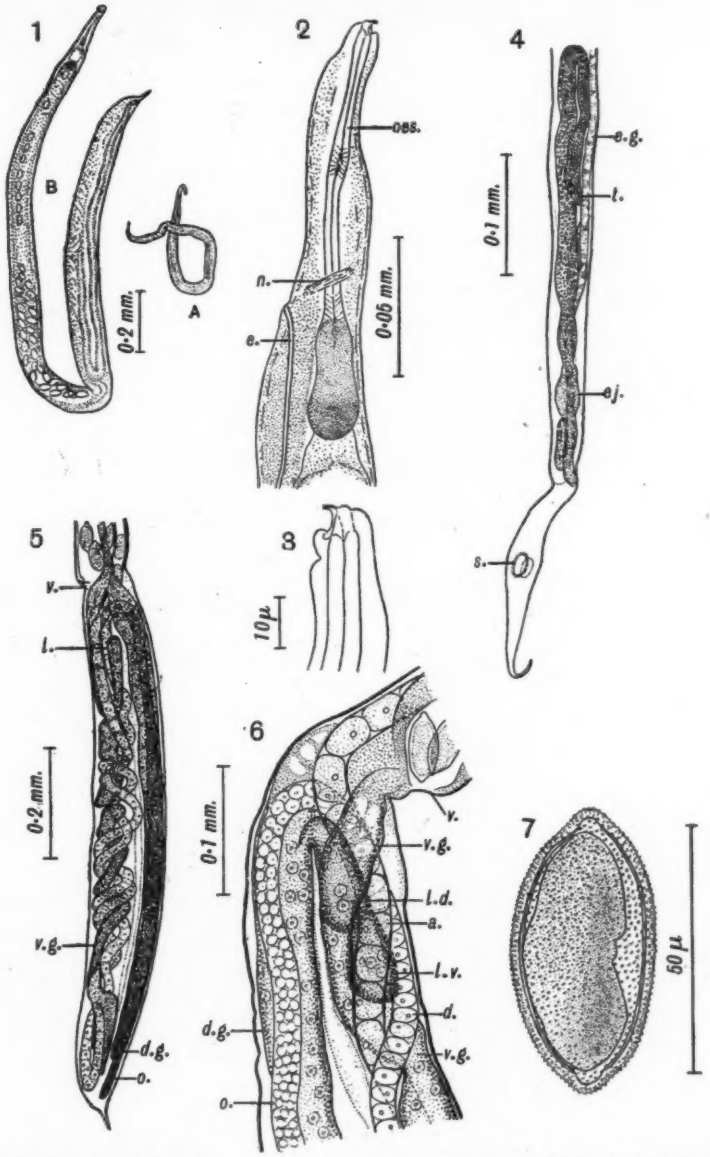
Of this species the collection contains several females and fragments of both sexes, but only one complete male. The latter measures (in balsam) about 0·98 mm. in length, and has a maximum thickness of about 0·04 mm. The females measure 2·06–2·97 mm. in length and 0·1–0·144 mm. in maximum thickness. The body tapers rather rapidly at the anterior end in both sexes, a great part of the prevulvar region in the female being of almost even thickness. In the male the tail (i.e. the region posterior to the cloacal aperture) tapers to a slender point and measures about 0·18 mm. in length. At about its middle it carries a pair of elliptical pits or 'suckers' (Fig. 4, *s.*). In the female the postvulvar region of the body is considerably stouter than the anterior region, and is rather suddenly narrowed near its extremity to form a short terminal spike.

The 'head' is exceedingly small. The mouth (Figs. 2, 3) opens dorsally. It has a thickened cuticular border, produced on its anterior (ventral) edge into a pair of delicate, pointed, spine-like processes which curve slightly over the aperture. The oesophagus, in both sexes, measures about 0·14–0·164 mm. in length. It has a well-marked posterior bulb, but for the greater part of its length is slender, though there is a very slight fusiform thickening a little in front of the middle of the slender portion. The nerve-ring is a little in front of the bulb. The intestine forms a wide, saccular expansion immediately behind its junction with the oesophagus, but (in the female at least) almost at once appears to become a very narrow tube, difficult to trace because it is obscured by the uterus. What is probably a continuation of the intestine can be detected, in favourable specimens, in the posterior region of the body, but here again it is difficult to trace among the other organs. No anus could be detected.* Little could be seen of the intestine in the male, owing to its extreme transparency. Just behind the nerve-ring and in front of the oesophageal bulb, on the ventral surface, there is a relatively large excretory pore, from which a conspicuous duct (Fig. 2, *e.*), with a thickened cuticular lining, can be seen running posteriorly for some distance. Unfortunately the material does not permit the further course of this duct to be traced, but possibly it is connected with two large glands in the posterior part of the body, which will be described below.

The genital organs of the male are extremely simple. No spicules or accessory piece can be detected. The cloacal aperture is situated on a slight protuberance of the body-wall, capable, no doubt, of forming a papilla-like plug which can be inserted in the vulva of the female. The sexes have not been observed *in copula*. The genital tube (Fig. 4) consists of a single, reflexed testis and a straight duct which shows some faint indications of differentiation into regions functioning as seminal vesicle and ejaculatory duct.

In the female the genital organs appear somewhat complex, though their arrangement is merely a variant of the simple plan observed in other species to be described. The vulva is situated behind the middle of the body, at a distance of 0·86–1·22 mm. from the posterior end. Immediately behind it there is an indentation of the ventral body-wall, so that it opens posteriorly. At this point the body is sometimes doubled sharply upon itself ventrally (Fig. 1). There is a single ovary, the extremity of which is close to the posterior end of the body. It runs straight along the dorsal side of the body-cavity, and anteriorly merges into the oviduct. This forms a loop a little behind the level of the vulva, and from this point runs back by a very convoluted course nearly to the posterior extremity, where it forms another loop. This descending branch of the oviduct and its ascending branch, which runs a parallel course, are spirally twisted about each other and about one of the two elongate excretory [?] glands of which further mention will be made. Anteriorly the ascending branch straightens out, crosses over behind the vulva from the ventral to the dorsal side of the body, and then pursues a straight course through the anterior region of the body almost as far as the hinder end of the oesophagus. Here it merges into the uterus, which at this point forms a sharp loop and begins to run straight back to the vulva. At the anterior loop there is a special region of the uterus which appears to function as a receptaculum seminis. The uterus proper has a thick, apparently glandular wall which closely invests the eggs. These are relatively numerous and, though arranged in a single row anteriorly,

* No anal aperture has been observed in the females of any of the four species of *Synoecnema* described in this paper, or in those of the other forms dealt with. The presence of an anus in the female of *S. fragile* is mentioned by de Magalhães (1905), but this author also describes the male as possessing separate copulatory and anal apertures—a feature the improbability of which has been pointed out by Pierantoni (1916).



Synoecnema hoplochaetellae

Fig. 1. A, male, and B, female, drawn to the same scale.

Fig. 2. Anterior end of female, viewed from left side. *e.* excretory duct; *n.* nerve-ring; *oes.* oesophagus.

Fig. 3. Anterior end of female, highly magnified, viewed from right side.

Fig. 4. Posterior portion of male, viewed from right side. *e.g.* excretory (?) gland; *ej.* ejaculatory duct; *s.* 'sucker'; *t.* testis.

Fig. 5. Posterior portion of female, viewed from left side. *d.g.* dorsal excretory (?) gland; *l.* lobe of ventral gland; *o.* ovary; *v.* vulva; *v.g.* ventral excretory (?) gland.

Fig. 6. Portion of female in vulvar region, viewed from right side. *a.* ascending limb of oviduct; *d.* descending limb of oviduct; *d.g.* dorsal excretory (?) gland; *l.d.* lobe of dorsal gland; *l.v.* lobe of ventral gland; *o.* ovary; *v.* vulva; *v.g.* ventral excretory (?) gland.

Fig. 7. Egg.

usually form two rows more posteriorly. Various successive phases of the development of the shell are seen from the anterior to the posterior end of the column of eggs, the last fifteen or more of which may have fully-formed shells. These (Fig. 7) are somewhat lemon-shaped, measure 0·046–0·06 × 0·024–0·034 mm., and have a relatively thick outer coat with a vertically striated or mammillated appearance. The contents of the eggs are unsegmented *in utero*. The proportions of the eggs change somewhat as they become more mature, those nearest to the vulva tending to become longer and narrower. In fact, the width appears to be almost in inverse proportion to the length; thus a series of eggs gave approximately the following measurements (in μ): 46 × 32, 48 × 34, 50 × 34, 50 × 30, 52 × 30, 52 × 28, 54 × 28, 56 × 26, 58 × 28, 58 × 26, 60 × 24.

A large part of the body-cavity of the female, in the posterior region, is occupied by two elongate, granular, multinucleate organs (or possibly two portions of one organ). These may possibly be connected with the excretory system, and are very closely associated with the different portions of the genital tube. One of them (Fig. 5, *d.g.*) is dorsal in position, and closely invests the ovary for almost the whole of its length. The other (Fig. 5, *v.g.*) is ventral, and partakes of the spiral coiling of the descending and ascending branches of the oviduct, with which it is associated. In some specimens it appeared possible to detect a junction of these two glands on the left side of the body in the vulvar region, but this remains somewhat doubtful, and all attempts to trace their ultimate connexion with the excretory duct in the anterior region unfortunately failed. The glands appear, in the main, to be simple band-like structures, but occasionally they give off lobes or branches in their course, particularly near their anterior ends, filling interstices between the other organs (Fig. 5, *l.*; Fig. 6, *l.d.*, *l.v.*). Similar glands appear to be present in the male (Fig. 4, *e.g.*), but their course could not be followed out in detail.

Synoecnema drawidae, n.sp.

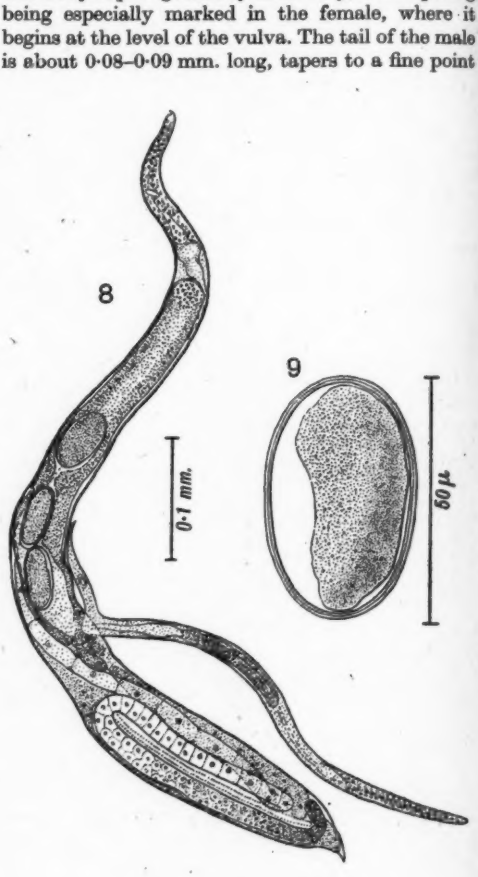
(Figs. 8, 9).)

Host. Drawida sp.*

Localities. (*a*) Srivaikuntam, Tinnevelly District, South India; (*b*) Sagar, Mysore State..

The former set of specimens, consisting of six pairs of worms *in copula* and two females, were taken from the coelomic cavities of segments vi–xvi of the hosts. The second set, consisting of two pairs and one female, came from the coelomic cavities of three juvenile specimens of what was probably the same species of earthworm.

This is a much shorter and relatively much stouter form than the preceding. The male measures (in balsam) about 0·47–0·56 mm. in length and about 0·018–0·028 mm. in maximum thickness, the female 0·81–1·36 and 0·066–0·1 mm. respectively. The specimens in the second set are a little larger and apparently more mature than those in the first. The body tapers gradually anteriorly, this tapering being especially marked in the female, where it begins at the level of the vulva. The tail of the male is about 0·08–0·09 mm. long, tapers to a fine point and bears a pair of pits or 'suckers' at about its middle. The postvulvar region of the body in the female is relatively stout, and tapers rather suddenly behind to a conical point.



Synoecnema drawidae

Fig. 8. Male and young female *in copula*.
Fig. 9. Egg.

The characters of the anterior end, so far as they can be made out, do not appear to differ materially from those in *S. hoplochaetellae*. The oesophagus is extremely difficult to see, owing to the presence of a mass of cells surrounding it. It appears, however,

* *D. dolosa* Gates *in litt.* (1936).

to be about 0·11–0·13 mm. long, and to be very narrow except at its hinder end, which is expanded into a small bulb. The nerve-ring is at about its middle. An excretory pore has not been clearly seen, probably owing to the mass of cells already mentioned, but in some specimens there appears to be a faint indication of it immediately behind the nerve-ring. As in *S. hoplochaetellae*, the intestine seems to begin with a wide expansion, which might almost be called a stomach, and then to be reduced to a narrow tube which, so far as can be made out, ends blindly near the hinder end of the body.

The genital tube of the male resembles that in *S. hoplochaetellae*, and no spicular structures can be detected. The cloacal aperture is situated on a protuberance which appears (Fig. 8) to function as an intromittent organ.

The female genital apparatus appears simpler than in *S. hoplochaetellae*, because the oviduct has almost straight descending and ascending branches instead of being spirally coiled. In general plan, however, the arrangement of the parts is the same. The vulva is situated considerably behind the middle of the body, at about 0·31–0·58 mm. from the posterior end, according to the size of the specimen. The uterus is similar in structure to that of *S. hoplochaetellae*, but contains relatively few eggs, arranged in a single row, of which only from two to six have fully-formed shells. These (Fig. 9) are oval, relatively thin and apparently without ornamentation or polar differentiation. They measure, when mature, 0·046–0·058 × 0·022–0·033 mm. The extremity of the ovary may extend into the conical 'tail', or may be reflexed as shown in Fig. 8. Multinucleate glands, similar to those described for *S. hoplochaetellae*, are present in the hinder part of the body, at least in the female, and have similar associations with the ovary and oviduct.

Synoecnema perionychis, n.sp.

(Figs. 10–14.)

Hosts. (*a*) *Perionyx* sp. (coelomic cavity); (*b*) *Perionyx* sp. (coelomic cavities of anterior segments of immature specimens).

Localities. Assam: (*a*) Dumpep, Khasi Hills; (*b*) Khezhabama, Naga Hills.

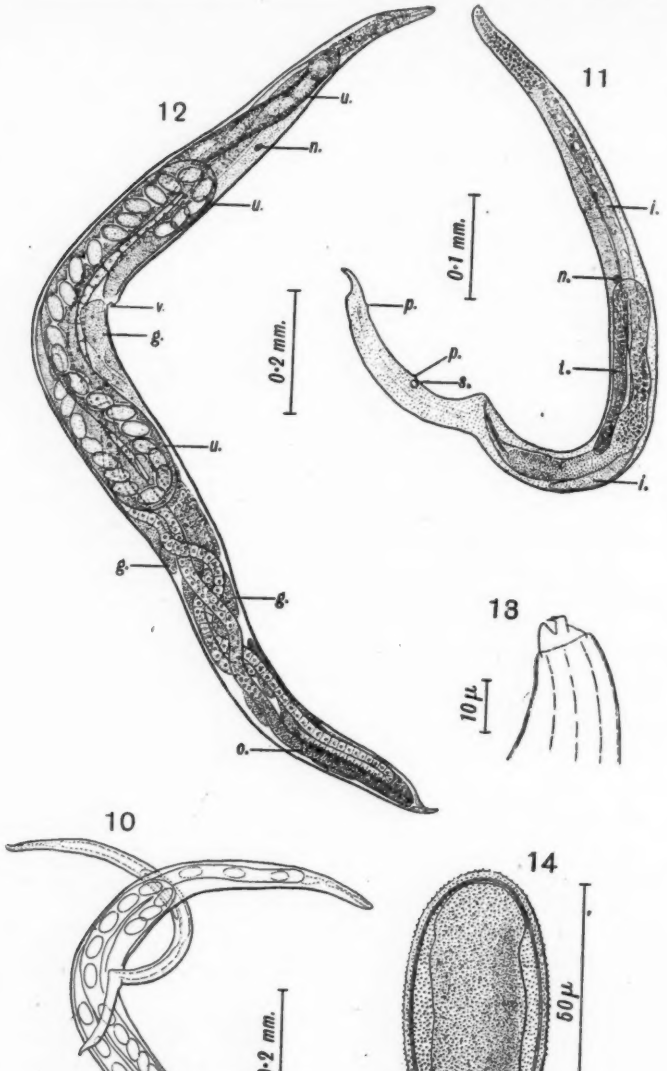
The two sets of this species contain a considerable number of specimens, most of which are pairs of worms *in copula*. In the typical attitude of copulation (Fig. 10) the body of the male is almost at right angles to that of the female, and curved round it towards its dorsal side. In one instance two males were attached, side by side, to the same female.

Males measure (in balsam) 0·55–1 mm. in length and 0·032–0·05 mm. in maximum thickness; females 1·35–2·36 and 0·066–0·156 mm. respectively. The body tapers gradually towards both ends. The tail of the male is almost cylindrical for the greater part of its length, but usually rather suddenly constricted near the tip. It measures 0·18–0·24 mm. in length. In some specimens a pair of 'suckers' or caudal pits appear to be present, or a single pit may be visible (Fig. 11). In the majority, however, these organs have not been detected. When visible, they appear as clear spaces of rounded or oval shape, and their position is very variable. One or both pits may be situated near the cloacal aperture, near the extremity of the tail, or anywhere between these points. In addition there are, in many specimens, some faint indications of caudal papillae, but it has not been possible to determine the precise number or arrangement of these, and indeed it is doubtful whether they have any constant number or position. The caudal extremity of the female, like that of the male, is rather suddenly narrowed to form a short, blunt, terminal process.

The 'head' (Fig. 13) appears to be somewhat retractile. Its exceedingly small size renders its characters very difficult to make out, even under the highest magnification. The usual paired spine-like ventral processes appear to be carried on a relatively large basal structure, with which they form a right angle, and they seem to be opposed by an unusually prominent and somewhat conical dorsal process or 'lip'. The oesophagus, in both sexes, measures 0·1–0·114 mm. (exceptionally 0·122 mm.) in length. It is greatly obscured by a mass of cells surrounding it, but its anterior half appears to be more or less cylindrical and wider than the succeeding portion, which is expanded behind into a bulb. The intestine has a degenerate and vacuolated appearance. In the male it seems to end blindly, on the dorsal side of the body-cavity, some distance in front of the cloacal aperture. In the female its posterior course could not be traced owing to the predominance of the genital organs. It is not expanded, but rather narrowed, at its junction with the oesophagus. An inconspicuous excretory pore is present at the level of the oesophageal bulb.

The genital organs of the male have the same arrangement as in other species of the genus, and no spicular structures appear to exist. The cloacal aperture is situated, as usual, on a ventral protuberance.

The genital tube of the female is arranged on essentially the same plan as in *S. hoplochaetellae* and *S. drawidae*, but this plan is modified by the presence of an anterior and a posterior loop in the uterus. The coiling of the oviduct is more complex than in *S. drawidae*, but less so than in *S. hoplochaetellae*. The ovary is almost straight, and ends near the posterior extremity. The vulva, which has a prominent anterior lip, is situated considerably in front of the middle of the body, at a distance of 0·57–0·92 mm. from the anterior end. This distance



Synoecnema perionychis

Fig. 10. Male and female *in copula*.

Fig. 11. Male, viewed from left side. *i*. intestine; *n*. nucleus of ventral gland; *p*., *p*. caudal papillae; *s*. caudal 'sucker'; *t*. testis.

Fig. 12. Female, viewed from right side. *g*., *g*. portions of multinucleate glands; *n*. nucleus of ventral gland; *o*. ovary; *u*. uterus; *v*. vulva.

Fig. 13. Anterior end of female, highly magnified, viewed from right side.

Fig. 14. Egg.

increases in direct proportion to the total length of the body. In specimens up to 1·52 mm. long it remains at about 0·65 mm., but increases fairly steadily in larger individuals. After running forward for a short distance, the uterus turns to run posteriorly, forms a loop some distance behind the vulva, and thence runs forward again to its junction with the oviduct, not far behind the junction of the oesophagus and intestine. At this point there is, as in other species, a short region acting as a receptaculum seminis. According to the age of the specimen, the uterus may contain from three to twenty-three eggs with fully-formed shells, and a number of others in earlier stages. As a rule the eggs are arranged in a single row, but in specimens with thirteen or more fully-formed eggs a double row sometimes begins to form between the anterior and posterior loops of the uterus. The shells of the eggs, which measure 0·046–0·058 × 0·024–0·032 mm., are of a very regular ovoid shape and thick, with the outer layer very closely and finely mammillated. In a drawing (Fig. 14) this delicate ornamentation can only be very coarsely represented.

Much of the postvulvar region of the body of the female appears to be occupied by multinucleate glands (Fig. 12, *g.*) similar to those described for *S. hoplochaetellae*. Lobes of these organs can be seen filling up many of the spaces between the coils of the genital tube. In both sexes a granular structure (? excretory gland), apparently containing one large nucleus (Figs. 11, 12, *n.*), can also be seen running along the ventral side of the body-cavity in the anterior region.

Synoecnema pheretimae, n.sp.

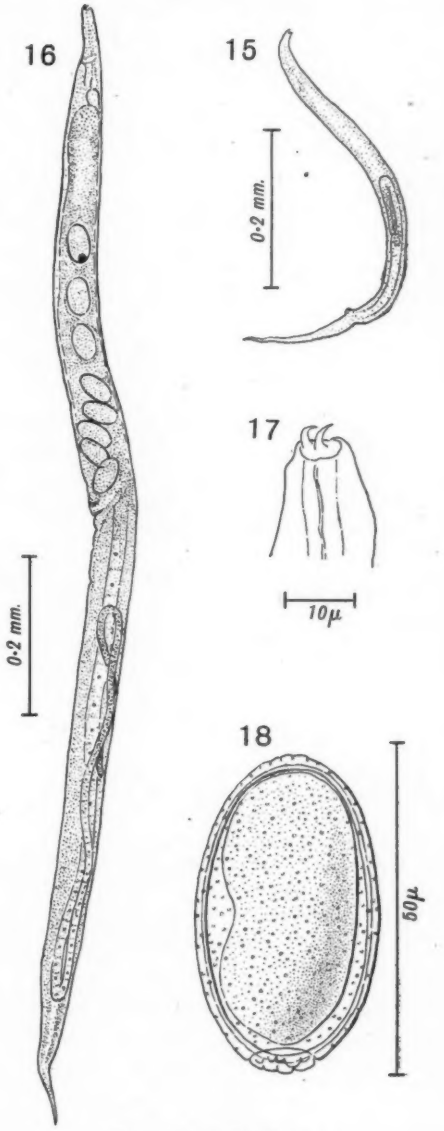
(Figs. 15–18.)

Host. Pheretima procera Gates, 1937* (coelomic cavities of anterior segments).

Locality. Singapore.

The material of this species, consisting of one male, several females and some fragments, was studied only in unstained glycerine preparations. The male measures about 0·58 mm. in length and 0·028 mm. in maximum thickness, the mature females 0·93–1·45 and 0·046–0·06 mm. respectively. An immature female measures only 0·6 mm. in length. The body, in both sexes, is of almost uniform thickness for the greater part of its length, tapering rather rapidly in front and more gradually behind. The posterior extremity forms a slender point. The tail of the male is 0·145 mm. long. No caudal 'suckers' have been observed. In the female the prevulvar region is very slightly stouter than the part of the body behind the vulva, but the vulva is not prominent.

The 'head' (Fig. 17) is exceedingly small, but the ventral mouth-hooks appear somewhat larger and stouter than in *S. hoplochaetellae*. The form of the oesophagus is difficult to make out, but it appears



Synoecnema pheretimae

Fig. 15. Male, viewed from left side.
Fig. 16. Female, viewed from left side.
Fig. 17. Anterior end of female, highly magnified; ventral view.
Fig. 18. Egg.

* *Bull. Raffles Mus., Singapore*, **13**, 193.

to have a slight posterior bulb and measures about 0·1–0·112 mm. in length. The nerve-ring is situated a little behind its middle. An excretory pore and duct, almost as conspicuous as in *S. hoplochaetellae*, are present, the pore being immediately behind the nerve-ring. Similar glands also appear to occupy a large part of the body-cavity in the postvulvar region of the female, but details of their arrangement could not be made out.

The genital organs of the male appear to be similar to those described for the three preceding species. No spicules could be detected. The cloacal aperture is situated on a papilla-like protuberance.

The termination of the ovary, in this species, is at about the middle of the postvulvar region, and not near the hinder extremity, and the forward loop of the oviduct is some distance behind the vulva. The latter is in front of the middle of the body, at 0·74–0·8 mm. from the posterior end. In other respects the arrangement of the female organs is the same as in *S. hoplochaetellae*. The uterus, in mature females, usually contains from eight to ten eggs in a single row, the four or five nearest to the vulva having fully-formed shells. These (Fig. 18) measure 0·048–0·05 × 0·026–0·03 mm. They show rather scattered vertical pore-like ornamentations, which appear as minute refringent dots in surface view. At the pole turned towards the vulva there is an area resembling a cap or operculum, with relatively large mammillations on its surface. At the opposite pole the contour of the shell is slightly irregular. Eggs in which the shell is not yet fully formed tend to be rather larger, especially in width. They measure 0·048–0·052 × 0·029–0·032 mm.

Genus **Perodira**,* nov.

Diagnosis. Drilonematidae. Mouth terminal, unarmed, leading into a small buccal cavity. Oesophagus relatively wide anteriorly and narrower, but expanding into a bulb, posteriorly. A pair of pouch-like lateral cervical organs and a pair of lateral caudal pits or 'suckers' present in both sexes. Tail of male blunt. A wide membranous ala [? pair of alae] present throughout the greater part of the body in the male. Spicules and accessory piece apparently absent. Vulva behind middle of body. Female genital tube single, anterior, reflexed.

Hab. Free in the coelomic cavities of earthworms. *Genotype. P. alata*, n.sp.

Perodira alata, n.sp.

(Figs. 19–26.)

Hosts. (*a*) One set of specimens, consisting of three males, three females and some fragments, from *Hoplochaetella anomala* Stephenson, 1920 (coelomic cavities of anterior segments); (*b*) three females and a fragment from *Drawida* sp.* (coelomic cavities).

Localities. Mysore State, South India: (*a*) South bank of Sharavati river, Jog falls; (*b*) Bababudan Hills (jungle between Kemmangundi and Kalhattagiri).

The specimens from *Hoplochaetella*, which are taken as the types, are mature and in fairly good condition. Those from *Drawida*, though of similar dimensions and probably of the same species, are less mature and in a somewhat poor state of preservation.

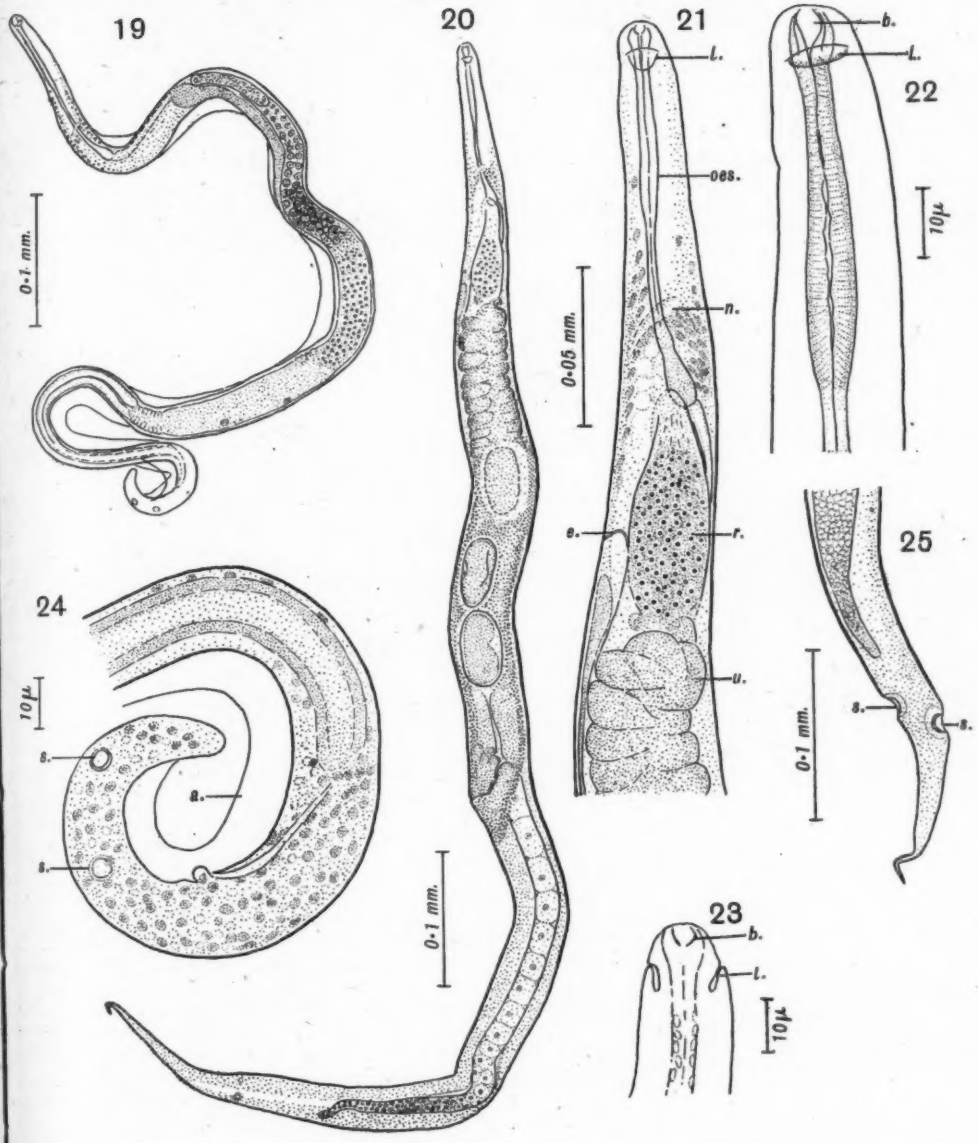
The males measure 0·84–1·1 mm. in length and 0·03–0·034 mm. in maximum thickness (excluding the width of the ala); the females 1–1·33 and 0·05–0·056 mm. respectively. The anterior end of the body tapers very gradually to a rather blunt extremity. At a little distance from the anterior end there is a pair of relatively large, pouch-like lateral organs or 'amphids' (Figs. 21, 22, 23, *l.*) with forwardly-directed slit-like apertures. The mouth leads into a small, somewhat goblet-shaped buccal cavity (Figs. 22, 23, *b.*), surrounded by a slightly expanded anterior portion of the oesophagus. The latter organ measures 0·114–0·142 mm. in length. Its anterior portion is relatively wide and slightly muscular, its posterior portion narrow, but expanded behind into an elongate, narrow bulb. The intestine appears to be narrow, but is very difficult to observe, and no anal aperture has been detected. The nerve-ring surrounds the narrow part of the oesophagus just in front of the bulb. The excretory pore is some distance behind the oesophageal bulb, and is the opening of a narrow duct (Fig. 21, *e.*).

The body of the male is strongly coiled, especially at the caudal end. The tail (Fig. 24) measures about 0·07 mm. in length, and its tip is blunt. On the tail there is a pair of lateral pits or sucker-like organs, more or less circular in shape and usually asymmetrically placed. In the cloacal region there is a pair of delicate ventro-lateral alae (Fig. 24, *a.*). A wide, membranous ala or, possibly a pair of such alae, also runs throughout the greater part of the body. The male genital tube is single. The extremity of the testis is reflexed. Posteriorly portions of the tube appear to be differentiated as a voluminous seminal vesicle and a long ejaculatory duct. No spicules have been observed.

The body of the female is without alae, and tapers posteriorly to a slender point. At a distance of about 0·1 mm. (more or less) from the caudal end there is a pair of lateral pits. These are very inconspicuous in the specimens from *Hoplochaetella* (Fig. 20), but much larger and more striking in those from *Drawida* (Fig. 25, *s.*). The genital tube is single and shows a very simple arrangement. The

* πήρα, pouch; δειρή, neck.

* *D. ampullacea* Gates, *in litt.* (1936).



Perodira alata

Fig. 19. Male. Fig. 20. Female, viewed from left side.
Fig. 21. Anterior end of female, viewed from left side. *e.* excretory duct; *l.* lateral organ; *n.* nerve-ring; *oes.* oesophagus; *r.* receptaculum seminis; *u.* uterus.
Fig. 22. Anterior end of female, highly magnified; lateral view. *b.* buccal cavity; *l.* lateral organ.
Fig. 23. Anterior end of male, highly magnified; dorso-ventral view. *b.* buccal cavity; *l.* lateral organ.
Fig. 24. Posterior end of male; lateral view. *a.* ala; *s.*, *s.* 'suckers'.
Fig. 25. Posterior end of female; dorso-ventral view. *s.*, *s.* 'suckers'.

ovary terminates near the posterior end, and is usually straight, though its extreme tip is occasionally reflexed. It passes into an oviduct which runs forward as far as the oesophageal bulb, and there doubles upon itself to join the uterus, the portion of which immediately behind the bend forms a receptaculum seminis (Fig. 21, *r.*). The uterus proper has thick, sacculated walls, and runs straight back to the vulva, which is situated slightly behind the middle of the body, at 0·55–0·62 mm. from the posterior end. It contains only from two to five eggs at a time, and of these only from one

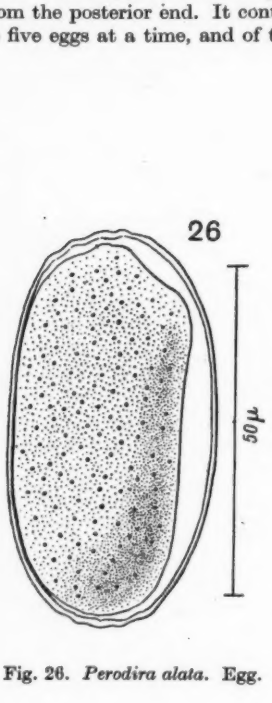


Fig. 26. *Perodira alata*. Egg.

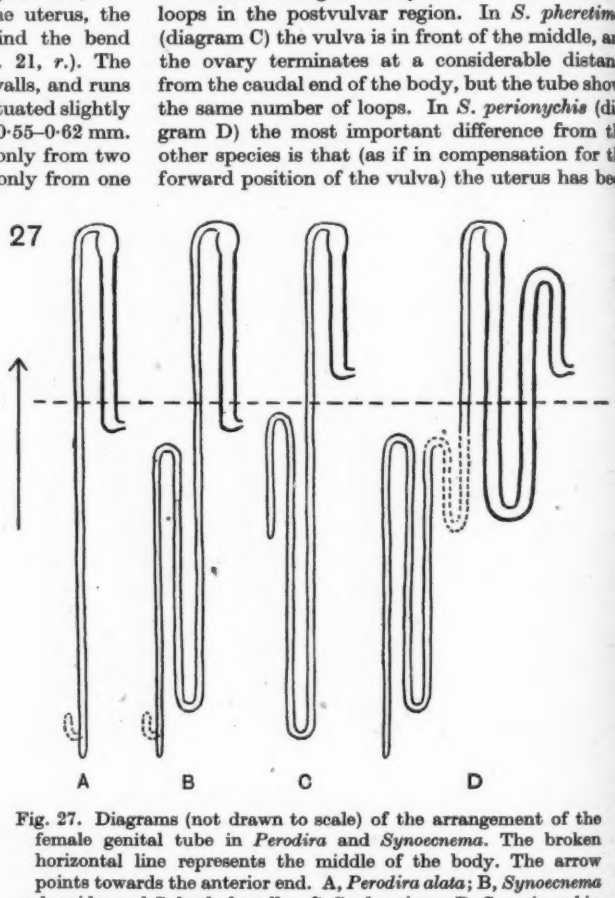


Fig. 27. Diagrams (not drawn to scale) of the arrangement of the female genital tube in *Perodira* and *Synoecnema*. The broken horizontal line represents the middle of the body. The arrow points towards the anterior end. A, *Perodira alata*; B, *Synoecnema drawidae* and *S. hoplochaetellae*; C, *S. pheretimae*; D, *S. perionychis*.

to three have fully-formed shells. The latter measure 0·058–0·066 × 0·028–0·038 mm. They are thin and without ornamentation, but slightly irregular in contour at the poles. Their contents are undifferentiated.

In the five species just described there is an interesting series of modifications in the antero-posterior looping of the female genital tube (Fig. 27). The simplest arrangement is that found in *Perodira alata* (diagram A). Here the vulva is behind the middle of the body, and the ovarian tube runs straight back from the anterior flexure to the caudal end. The extreme tip of the ovary may be straight or slightly reflexed. In *Synoecnema drawidae* and *S. hoplochaetellae* the arrangement, reduced to its essential plan, may be represented as in diagram B. The vulva is still behind the middle, but the ovarian tube has been lengthened by the introduction of two loops in the postvulvar region. In *S. pheretimae* (diagram C) the vulva is in front of the middle, and the ovary terminates at a considerable distance from the caudal end of the body, but the tube shows the same number of loops. In *S. perionychis* (diagram D) the most important difference from the other species is that (as if in compensation for the forward position of the vulva) the uterus has been lengthened by being looped twice upon itself. The arrangement of the ovarian tube is essentially the same as in diagram B, but in some specimens two short additional loops appear in it, as shown by the interrupted outlines. This may merely indicate that a lengthening of the tube takes place in the more mature individuals. It is interesting to note that the arrangement of the genital tube is probably of type D in the genotype of *Synoecnema*, *S. fragile*. The figure given by de Magalhães (1905, Fig. 4 (1)) shows a posterior loop in the uterus, though the interpretation of the course of the tube in this figure seems likely to be incorrect, since it is shown apparently running posteriorly at first from the vulva.

Family CREAGROCERCIDAE, nov.

For the present the characters of this family must be taken as the same as those of its type and only genus:

Creagrocercus,* n.g.

Diagnosis. Small, relatively stout forms with anterior end curled ventrally. Anterior end with a pair of latero-ventral processes ending in flagella, and a pair of circular lateral cervical organs. A shallow buccal capsule present. Oesophagus club-shaped and muscular. Intestine apparently ends blindly. Tail in both sexes bears at its tip a minute hook. Male with a pair of unequal and dissimilar spicules. Genital tubes paired and opposed in both sexes; outstretched in male, reflexed in female. Spermatozoa filiform. Vulva at about the middle of the body.

Hab. Coelomic cavities of an earthworm.
Genotype. C. barbatus, n.sp.

Creagrocercus barbatus, n.sp.

(Figs. 28–34.)

Host. Moniligaster gravelyi Stephenson† (coelomic cavities of anterior segments).

Locality. Kavalai, Cochin, South India.

This curious form is represented in the collection by several specimens of both sexes. There is little difference in size between males and females, the former measuring 1·38–1·55 mm. in length and 0·09–0·11 mm. in maximum thickness (under slight pressure), the latter 1·275–1·9 and 0·1–0·13 mm. respectively. The body is usually much coiled, the anterior end being invariably curled towards the ventral side. The 'head' (Figs. 28, 29, 34) bears a pair of small circular lateral organs or 'amphids', and a pair of finger-shaped latero-ventral processes, each of the latter ending in a slender flagellum. On the ventral surface, at about 0·05 mm. from the anterior extremity, there is a slight transverse ridge or protuberance of the cuticle (Figs. 28, 29, *r.*). At about the same distance behind this there is a second very slight eminence on which the excretory pore appears to open. The posterior end of the body, in both sexes, tapers gradually and bears at its blunt tip a minute hook (Figs. 30, 32). The caudal extremity, with its hook, somewhat resembles in shape the end of an elephant's trunk.

There is a shallow buccal capsule, with slightly thickened cuticular lining. The oesophagus is simple, club-shaped and muscular, and measures 0·15–0·17 mm. in length. It is surrounded by the nerve-ring at about its middle. The intestine appears to end blindly in the posterior region of the body. No anal aperture has been detected in the female. The caudal region, in both sexes, is occupied by a granular structure (Figs. 28–30, *c.g.*) which appears invariably to contain two large nuclei, and is probably a gland connected with the terminal caudal organ. Behind the excretory pore there is also a nucleated granular structure, probably an excretory gland (Figs. 28, 29, *g.*).

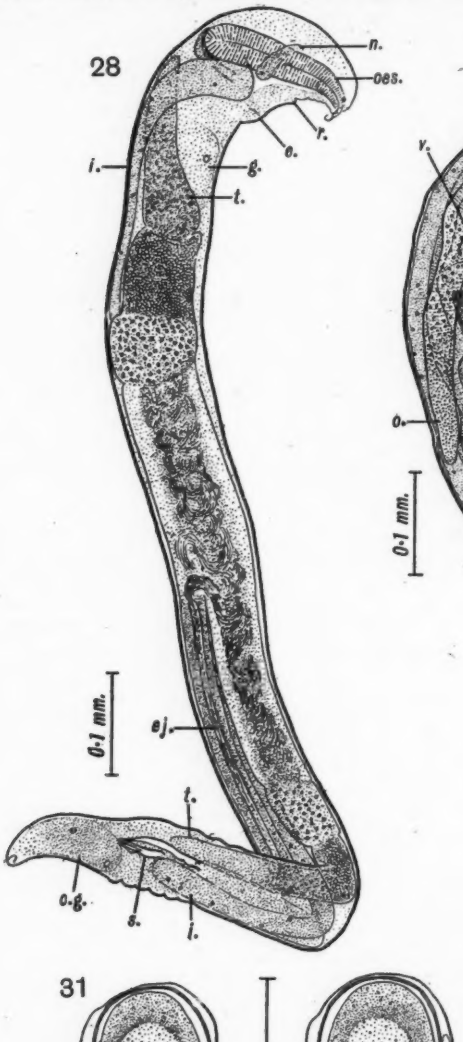
In both sexes the genital organs are paired and directly opposed. In the male the two testes are straight, the anterior testis extending almost as far as the hinder end of the oesophagus, the posterior to the cloacal region. The proximal portions of the two organs form voluminous reservoirs for spermatozoa. The latter are remarkable in that they are filiform, and not amoeboid as in most nematodes. The relatively thick-walled ejaculatory duct (Fig. 28, *ej.*) runs parallel to the posterior testis, and in most specimens is hidden by it. The specimen figured displays the structure exceptionally well owing to an accidental torsion of the posterior half of the body about its axis. There is a pair of unequal and dissimilar spicules, the form of which is indicated in Figs. 30 and 33. The left spicule measures (in a straight line from root to tip) 0·08–0·09 mm., the right 0·046–0·06 mm. The former has a slightly knobbed tip, while the latter is apparently tubular. Both spicules appear to be of rather complex structure. The cloacal aperture is at about 0·12–0·15 mm. from the posterior extremity.

The vulva may be slightly in front of or slightly behind the middle of the body, and opens directly into the uterus, the two branches of which form a continuous tube. At its extremity each branch is connected with a reflexed oviduct, and the straight ovaries cross each other to end respectively in front of and behind the vulva. In the thick-walled uterus the eggs are arranged in a single row, separated by considerable intervals in which the lumen is narrowed and usually filled with spermatozoa. The eggs (Fig. 31) measure about 0·044–0·054 × 0·028–0·04 mm. The uterus contains at one time 15–18 eggs, of which about 7–12 have fully-formed shells. The shell proper is thin, smooth and roundish-oval, but is covered externally with a coat of hyaline material which usually shows extensive and irregular thickenings. The granular contents of the eggs frequently show one or two large vacuoles.

This curious form has so many peculiarities that it appears impossible to assign it to any known family. With reluctance, therefore, the writer has proposed the erection of a new family for a single species. At the same time it is difficult to determine what position this family should occupy in a general scheme of classification, and no attempt is made at present to decide this question. There seems, at any rate, to be no evidence that *Creagrocercus* has any

* κρεάγρα, flesh-hook; κέρκος, tail.

† *M. vaillanti*, n.sp., Gates, *in litt.* (1936); but see Gates, 1940, *Rec. Ind. Mus.* **42**, 504.



Creagrocercus barbatus

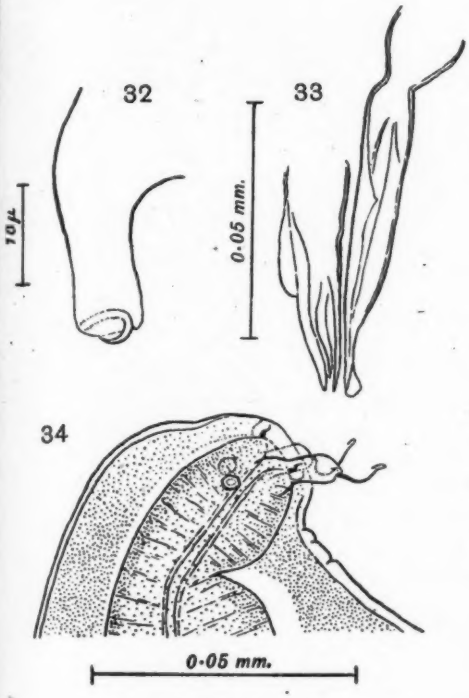
Fig. 28. Male; anterior portion in lateral view, posterior portion in latero-ventral view. *c.g.* caudal gland; *e.* excretory pore; *ej.* ejaculatory duct; *g.* excretory (?) gland; *i.* intestine; *n.* nerve-ring; *oes.* oesophagus; *r.* ventral ridge; *s.* spicules; *t.*, *t.* testes.

Fig. 29. Female, viewed from right side. *c.g.* caudal gland; *e.* excretory pore; *g.* excretory (?) gland; *i.* intestine; *o.*, *o.* ovaries; *r.* ventral ridge; *v.* vulva.

Fig. 30. Posterior end of male; lateral view. *c.g.* caudal gland; *l.* left spicule; *r.* right spicule.

Fig. 31. Eggs.

close affinity to the Drilonematidae, where, on account of its habitat, one might have expected to find its nearest relatives.



Creagrocercus barbatus

Fig. 32. Caudal extremity of male, highly magnified.
Fig. 33. Spicules of male; ventral view, highly magnified.
Fig. 34. Anterior end of female, highly magnified, viewed from right side.

Family SCOLECOPHILIDAE, nov.

Diagnosis. Relatively short and stout nematodes with thin cuticle. Body tapering more gradually in front than behind. Mouth without recognizable lips. Oesophagus with muscular anterior portion and large, glandular posterior bulb. Intestine terminates blindly. Two equal spicules and an accessory piece present in the male [of *Scolecophilus*]. Vulva near the anterior end of the body. A single functional uterine branch, with ovary, running posteriorly. A blind sac, lying parallel to the anterior portion of the uterus and acting as an egg-reservoir, probably represents a second uterine branch. Eggs oval, with thin shells.

Scolecophiloides gatesi, n.g., n.sp.

(Figs. 35–38.)

Host. Perionyx sp. (coelomic cavity, apparently burrowing in tissues).

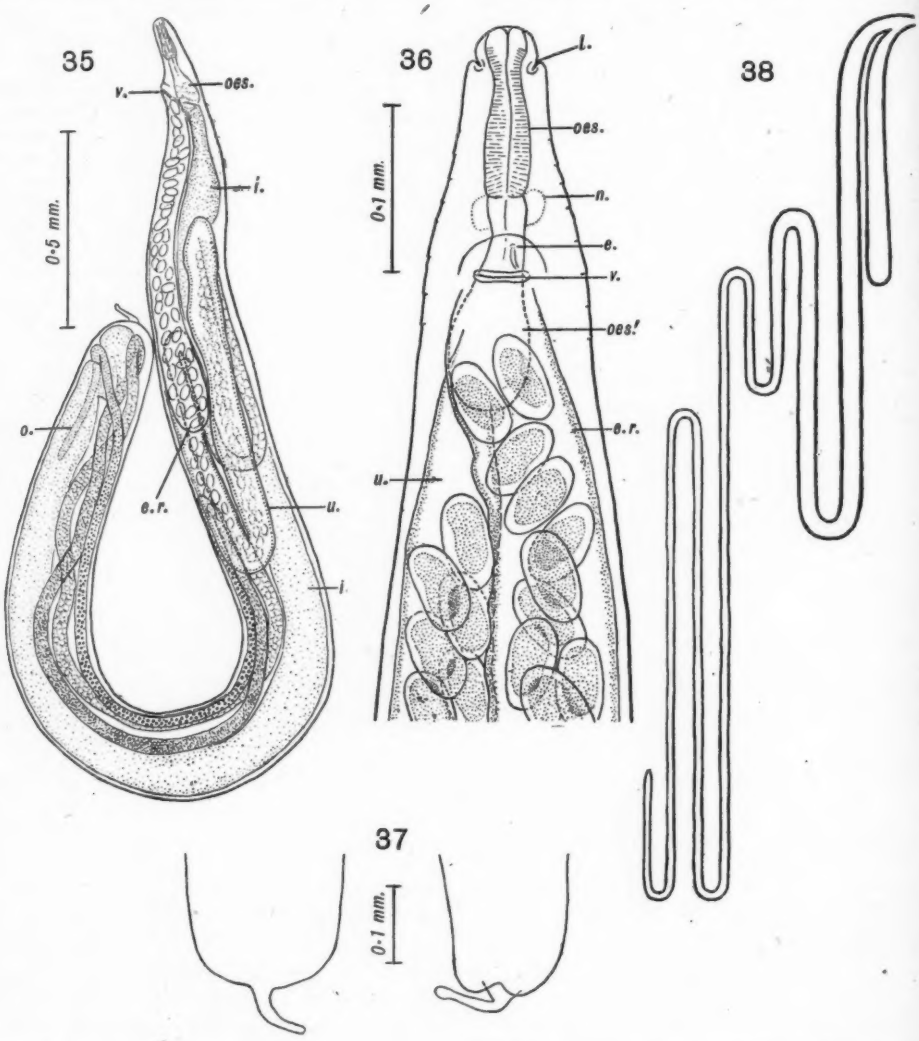
Locality. Dumpep, Khasi Hills, Assam.

Of this form the collection contains several females and fragments of females, but no males. Mature specimens measure 2·5–3·8 mm. in length (excluding the caudal appendage) and 0·25–0·3 mm. in maximum thickness, when slightly flattened under the pressure of a coverglass. The body tapers gradually towards the anterior end. Posteriorly it is bluntly rounded or almost abruptly truncate (Figs. 35, 37), but carries a finger-like terminal appendage about 0·07–0·11 mm. long. The cuticle is thin and smooth, but the rather prominent edges of a few transverse striations or annulations can be seen on the lateral surfaces near the anterior end. No cephalic papillae have been observed, but near the anterior extremity there is a pair of pit-like lateral organs (Fig. 36, *l.*). The mouth appears to be simple. The oesophagus measures about 0·2–0·24 mm. in length. It consists of a somewhat club-shaped, muscular anterior portion and a glandular posterior bulb with a long neck, the bulb and its neck together being longer than the muscular portion. The intestine is wide and apparently terminates blindly at the posterior end of the body. Faint indications of a nerve-ring could be detected in some specimens immediately behind the muscular portion of the oesophagus.

The vulva, which is a transverse slit (Fig. 36, *v.*) is situated at about 0·15–0·19 mm. from the anterior extremity, on a slight protuberance of the body-wall. Just in front of it, on the anterior surface of this protuberance, is the excretory pore. There is a single genital tube, arranged in a number of antero-posterior loops, as indicated in Fig. 35, and diagrammatically in Fig. 38, and terminating in an ovary the reflexed tip of which lies near the posterior end of the body. The uterus is confined to about the anterior half of the body, and forms a U-shaped loop. Lying parallel to its ascending limb, and opening with it at the vulva, but not extending back as far as the posterior loop, there appears to be a thin-walled, blind sac (Figs. 35, 36, *e.r.*), filled with eggs and possibly representing a rudimentary second uterine branch. The eggs have thin, oblong-oval shells with a slightly irregular surface, measuring about 0·052–0·062 × 0·026–0·032 mm. Their contents are granular and appear to be undifferentiated.

This worm bears strong resemblances to *Scolecophilus lumbricicola* Baylis & Daubney, 1922, which was recorded from the coelomic cavity of *Perionyx m'intoshi* in Nepal. In that species also a blind egg-

sac was described, lying parallel to the terminal portion of the uterus. The male of *Scolecophilus* has spicules of a very distinctive form, and it is unfortunate that males of the present species are not available for comparison with it. The female genital tube of *Scolecophilus* has a rather simpler arrangement, the uterus being straight instead of forming a loop. While the two forms may possibly be co generic, it seems advisable, pending the discover of the male of the present form, to treat this representing a distinct though closely related genu A new family has been erected provisionally abov for the reception of the two genera, but the syste matic position of the family is quite uncertain.



Scolecophiloides gatesi

Fig. 35. Female, viewed from left side. *e.r.* blind end of egg-reservoir; *i.* intestine; *o.* ovary; *oes.* oesophagus; *u.* uterus; *v.* vulva.

Fig. 36. Anterior end of female; ventral view. *e.* excretory pore; *e.r.* egg-reservoir; *l.* lateral organ; *n.* nerve-ring; *oes.* muscular portion of oesophagus; *oes'.* oesophageal bulb; *u.* uterus; *v.* vulva.

Fig. 37. Posterior ends of two females.

Fig. 38. Diagram (not drawn to scale) of arrangement of female genital organs.

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THREE NEW OPHIDIAN LUNG MITES OF THE SUBFAMILY ENTONYSSINAE EWING, 1922

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(With 14 Figures in the Text)

INTRODUCTION

In this paper certain aspects of the taxonomy of the subfamily Entonyssinae Ewing, 1922, are reviewed, and three new species of lung mites from snakes are described. Two of these new forms I am placing in the genus *Ophiopneumicola* Hubbard, 1938, while the remaining species is placed in the genus *Entonyssus* Ewing, 1922.

The subfamily Entonyssinae was created by Ewing on the basis of a single genus and species, *Entonyssus halli* Ewing, 1922. This subfamily was to include those dermanyssid mites with 'Both chelae of chelicera present, the fixed one harpoon-like, with a single recurved barb, and the movable one falciform; hypostome without recurved hooks.'

The generic diagnosis of *Entonyssus* included the above points, also 'legs all similar'.

In 1938 Hubbard emended the subfamily Entonyssinae so as to include the genus *Ophiopneumicola*, which he created at that time. This generic diagnosis differs from that of *Entonyssus* in that 'Chelae are gently curved and slightly recurved', and that legs are not all similar, as tarsus I is cylindrical, unlike those of other legs. The type species of this genus is *Ophiopneumicola colubri* Hubbard, 1938.

Eight species have been described for the Entonyssinae. These are:

(1) *Entonyssus halli* Ewing, 1922 [host: *Pituophis melanoleucus* (Daudin)]; (2) *Entonyssus rileyi* Ewing, 1924 [host: 'Rattlesnake']; (3) *Entonyssus glasmacheri* Vitzthum, 1935 [host: *Elaphe quadrivittata quadrivittata* (Holbrook)]; (4) *Entonyssus ewingi* Hubbard, 1939 [host: *Crotalus atrox* Baird & Girard]; (5) *Entonyssus vitzthumi* Schmidt, 1940 [host: *Pituophis sayi sayi* (Schlegel)]; (6) *Ophiopneumicola colubri* Hubbard, 1938 [host: *Coluber flagellum flavigularis* (Hallowell)]; (7) *Ophiopneumicola bedfordi* (Radford, 1937) [host: *Dendraspis angusticeps* Smith]; (8) *Ophiopneumicola hamertoni* (Radford, 1939) [host: *Thamnophis sirtalis sirtalis* (L.)].

The following new species are proposed:

(1) *Entonyssus heterodontos* n.sp. This species is based upon a total of thirty mites collected. Of these, twenty-three females, and six males were taken from the lung and trachea of a hognose snake, *Heterodon contortrix contortrix* (L.), and one female specimen from the trachea of a king snake, *Lampropeltis calligaster* (Harlan).

(2) *Ophiopneumicola natricis* n.sp. Forty-three specimens of this mite were collected, including thirty-nine females (adults), two female nymphs, one female larva, and one adult male. Of these, nine, sixteen, and eleven specimens respectively, were taken from three specimens of *Natrix sipedon pictiventris* Cope, and seven were taken from a *N. s. sipedon* (L.).

(3) *Ophiopneumicola elaphes* n.sp. Twenty-six specimens of this form, including twenty-five females and one male, were taken from two specimens of *Elaphe obsoleta obsoleta* (Say). Four of these were taken from an *Elaphe* from Iowa, and the remainder from a snake from Buffalo, New York.

All specimens were killed in hot Bouin's fluid, and mounted in a polyvenol alcohol, lactic acid, phenol medium. All specimens were collected by the writer.

DESCRIPTIONS OF NEW SPECIES

Entonyssus heterodontos n.sp.

A. *Female*

Colour white; body proper over twice as long as it is broad. Chelae are almost identical with those of *E. ewingi* in that the tip of the fixed chela is abruptly recurved ventrally, with a ventral subterminal enlargement, and a sharp, posteriorly directed point. Like *ewingi*, the fixed chela has a finger-like process, but this appears to lie lateral to the movable chela, rather than fitting into a 'keyhole' in it, as in the former species. There are nineteen pairs of spines on the dorsal shield. Sternal plate elongate, with two lateral projections, and three pairs of spines, borders genital aperture anteriorly, extends from posterior margin of coxa I to posterior margin of coxa III; genital plate elongate, narrow, rounded posteriorly, bears one pair of spines; a pair of small endopodal plates present; anal plate heart-shaped, bears three spines. Each half of labium trilobate; anterior margin of labrum bilobate, fimbriate; prominent mid-ventral suture in capitular shell; palpi 4-segmented, ultimate segment with a stout, furcate spine. Legs I, II, and III of about equal length, leg IV longest; tarsus I

cylindrical, of equal diameter throughout its length; pulvillus I bears two ring-like structures, 'ocelli'; spiracles dorsal to coxae IV.

Measurements of one of cotype females: total length (from anterior margin of labium) 960 μ; trunk length 847 μ, trunk width 380 μ, length: leg I, 601 μ; leg II, 601 μ; leg III, 601 μ; leg IV, 708 μ.

B. *Male*

Differs from female in following respects: Smaller size; modified chelicerae, as in males of *Entonyssus vitzthumi*, *E. ewingi*, *E. glasmacheri*, *Ophiopneumicola bedfordi*, and *O. hamertoni*, i.e. movable chela elongate, bifurcate; fixed chela short, straight; sternal and genital plates united to form a single ventral plate; body about twice as long as it is wide.

Measurements of a cotype specimen: total length 770 μ; trunk length 662 μ; trunk width 354 μ; length: leg I, 554 μ; leg II, 585 μ; leg III, 570 μ; leg IV, 631 μ.

Host: *Heterodon contortrix contortrix* (L.). Habitat: lung and trachea. Locality collected: Ottumwa, Iowa. Cotype specimens: two males, and six females in the United States National Museum.

Ophiopneumicola elaphes n.sp.

A. *Female*

Colour white; chelae very similar to those of *O. hamertoni*, in that fixed chela bears a tooth on its inner edge. Palpus 5-segmented, ultimate segment much smaller than penultimate; the latter bears a stout, furcate spine; anterior margin of each half of the labium trilobate, median lobes sharply pointed; anterior margin of labrum fimbriate. Dorsal shield with nineteen pairs of spines, and several smaller, unpaired spines; sternal plate with four lateral projections, and three pairs of spines; genital plate lacking spines; small endopodal plates present; anal plate with three spines. Legs I and II about equal in length, legs III and IV longer, leg IV longest; spiracles dorsal to coxae IV; tarsus of leg I cylindrical, of equal diameter throughout; pulvillus of leg I with two 'ocelli', as in *O. hamertoni* and *Entonyssus heterodontos*.

Measurements of a cotype female: total length 832 μ; trunk length 724 μ; trunk width 400 μ; length: leg I, 554 μ; leg II, 554 μ; leg III, 584 μ; leg IV, 662 μ.

B. *Male*

One male specimen was found. This was crushed in mounting, so that it was impossible to secure accurate measurements. Legs, pulvilli, labium, labrum, palpi, and spination of dorsal shield as in female; mouth parts modified, as in males of other species. It was impossible to trace the outline of the ventral plate.

Host: *Elaphe obsoleta obsoleta* (Say). Habitat: lung and trachea. Locality collected: Iowa City, Iowa, and Buffalo, New York. Cotypes: three females and one male, deposited in the United States National Museum.

Ophiopneumicola natricis n.sp.

A. *Female*

Colour white; body proper about 1½ times as long as it is broad. Tip of fixed chela slightly curved, but not recurved, overlaps movable chela anteriorly, tip of movable chela more sharply curved, fixed chela slightly the longer. Palpus 4-segmented, ultimate segment with several spines, including one very stout, furcate spine on its inner surface. Anterior margin of labrum fimbriate; anterior margin of labium possesses an outer pair of short, blunt, extensions, and an inner pair of more slender processes, labium possesses three pairs of spines. Dorsal shield bears sixteen pairs of spines, as well as several smaller, unpaired spines; sternal plate roughly rectangular, with four paired, lateral projections, near the anterior and posterior ends; genital plate pentagonal in outline, with one pair of spines; a pair of small endopodal plates present; anal plate with three spines. Legs I, II and III about equal in length; in some specimens leg I is slightly longer than II or III. Leg IV is the longest; spiracles dorsal to coxae IV. Each leg terminates with a pulvillus and a pair of claws; tarsus of leg I cylindrical; pulvillus of leg I with two 'ocelli'; claws of leg I about twice as large as those of other legs; spines present on all segments of all legs.

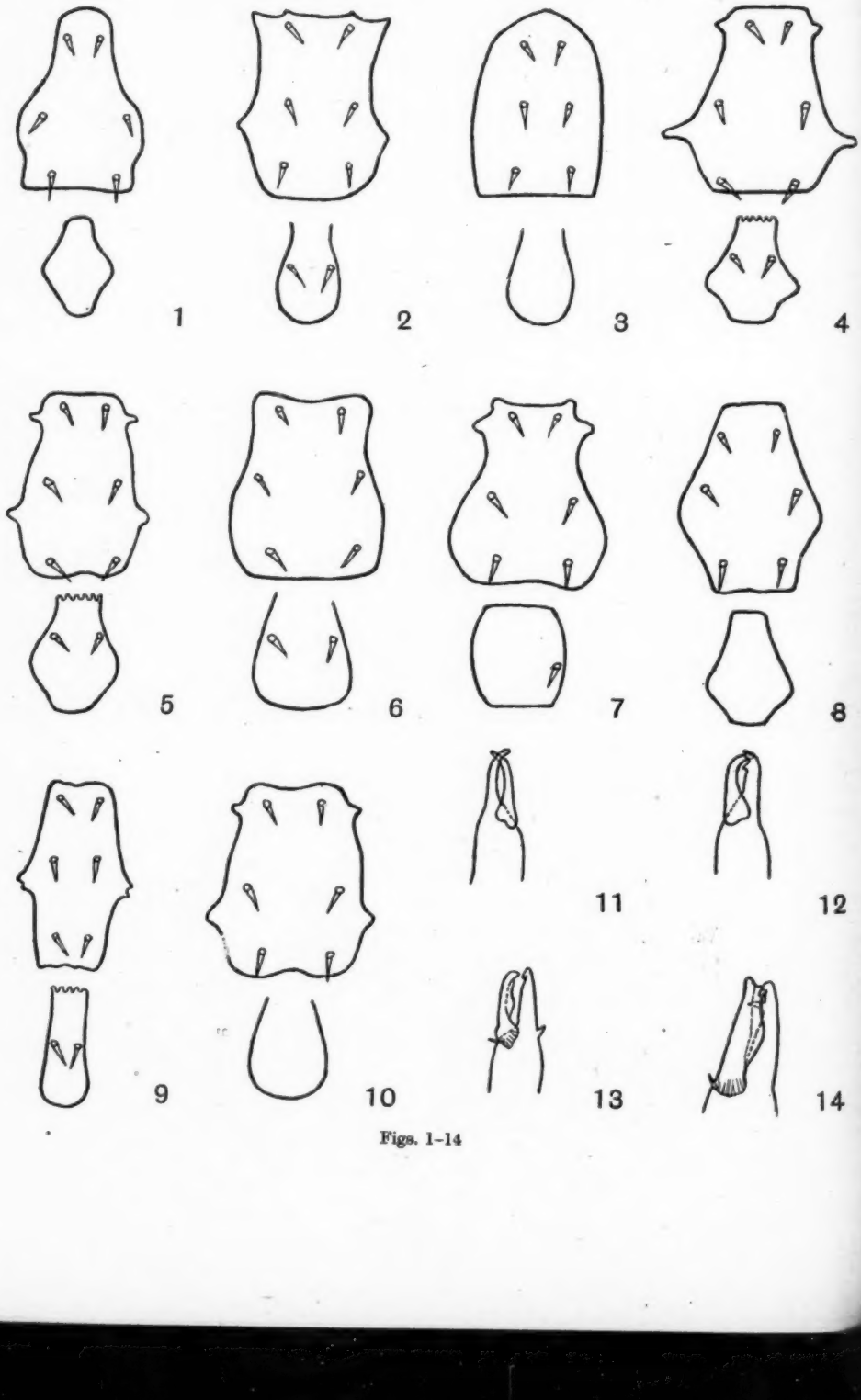
Measurements of a cotype female: total length 800 μ; trunk length 655 μ; trunk width 407 μ; length: leg I, 558 μ; leg II, 558 μ; leg III, 550 μ; leg IV, 631 μ.

B. *Male*

One male was taken from a *Natrix s. sipedon*. As the mite was slightly distorted, it was impossible to make out the outline of the ventral plate. Differs from female in smaller size, modified chelicerae (like those of males of other species), ventral plate (combined sternal and genital plates). Anal plate separate.

Measurements of cotype male: total length 585 μ; trunk length 493 μ; trunk width 293 μ; length: leg I, 385 μ; leg II, 462 μ; leg III, 447 μ; leg IV, 527 μ.

Host: *Natrix sipedon pictiventris* Cope. Habitat: lung and trachea. Locality collected: Ocala, Florida. Cotypes: three females and one male in the United States National Museum.



Figs. 1–14

KEYS TO THE ENTONYSSINAE

Because of the differences between male and female specimens of each of the species for which males have been described, it was thought best to set up a separate key for each sex. Males have been described for all of the members of the subfamily except *Entonyssus halli*, *E. rileyi* and *Ophiopneumicola colubri*.

Key to females

1. Chelicerae with fixed arm harpoon-like, being sharply pointed, and with a single recurved, distal hook. Genus **Entonyssus** 2
   Fixed arm of chelicerae not harpoon-like, lacking a recurved distal hook, distal end of fixed chela curved, slightly recurved. Genus **Ophiopneumicola** 7
2. Chelicerae stouter than palpi, movable chela almost straight, and with a rough cutting edge, thicker than fixed chela. **Entonyssus rileyi**
   Chelicerae not stouter than palpi, movable chela not straight, and lacking a rough cutting edge. 3
3. Colour brown, movable chela falciform. **E. halli**
   Colour white. 4
4. Chelicerae elaborate, lateral ala of fixed chela possessing a finger-like process. 5
   Fixed lacking a finger-like process. 6
5. Abdomen about twice as long as it is broad, pulvillus I lacking 'ocelli'. **E. ewingi**
   Abdomen about two and one half times as long as it is broad, pulvillus I possessing 'ocelli'. **E. heterodontos**
6. Ventral surface of fixed chela with two rounded serrations, tip of fixed chela bent at about a 90 degree angle, each half of labium trilobate. **E. vitzthumi**
   Ventral surface of fixed chela lacking serrations, fixed chela abruptly recurved at tip, each half of labium monolobate. **E. glasmacheri**
7. Pulvillus lacking at distal end of tarsus I. **O. bedfordi**
   Pulvillus present at distal end of tarsus I. 8
8. Pulvillus I lacking 'ocelli', legs I much shorter than legs II. **O. colubri**
   Pulvillus I possessing 'ocelli', legs I nearly equal in length with legs II. 9
9. Claws of tarsus I of equal size with those of tarsus II, inner edge of fixed chela with a single tooth. **O. elaphes**
   Claws of tarsus I at least twice as large as those of tarsus II, inner edge of fixed chela lacking a tooth. **O. natricis**

Key to males

1. Pulvillus present at distal end of tarsus I. 2
   Pulvillus lacking at distal end of tarsus I. **O. bedfordi**
2. Anal plate not separate from ventral plate. 3
   Anal plate separate from ventral plate. 4
3. Each half of labium trilobate. **E. vitzthumi**
   Each half of labium monolobate. **E. glasmacheri**
4. Pulvillus I lacking 'ocelli', anterior margin of medial lobes of labium fimbriate. **E. ewingi**
   Pulvillus I possessing 'ocelli'. 5
5. Anterior margin of each half of labium monolobate. **O. hamertoni**
   Anterior margin of each half of labium bilobate, or trilobate. 6
6. Claws of tarsus I of equal size with those of tarsus II. **O. elaphes**
   Claws of tarsus I larger than those of tarsus II. 7
7. Claws of tarsus I only slightly larger than those of tarsus II, length-width ratio of basal segment of tarsus I, in order of 4 : 1. **E. heterodontos**
   Claws of tarsus I at least twice as large as those of tarsus II, length-width ratio of basal segment of tarsus I in order of 7 : 3. **O. natricis**

SPECIES OF SNAKES EXAMINED FOR MITES

A total of 141 adult snakes, representing thirty-five species, were examined for lung mites. Thirteen individuals, representing eight species, were found to be infested. A detailed list of the specimens examined, together with the incidence of parasites, is given in Table 1.

SPECIES INCERTAE SEDIS

A. Twenty-three mites taken from three specimens of *Pituophis sayi sayi* (Schlegel), resemble *Entonyssus vitzthumi* in all respects but the following:

Legends to Figs. 1–14

Fig. 1. Sternal and genital plates of *Ophiopneumicola colubri*, adapted from Hubbard (1938), *Trans. Amer. Micr. Soc.* **57**, 400–6.
Fig. 2. Sternal and genital plates of *O. hamertoni*, adapted from Radford (1939), *Parasitology*, **31**, 243–54.
Fig. 3. Sternal and genital plates of *O. bedfordi*, adapted from Radford (1937), *North-West Nat.* **12**, 38–42.
Fig. 4. Sternal and genital plates of *O. elaphes* n.sp.
Fig. 5. Sternal and genital plates of *O. natricis* n.sp.
Fig. 6. Sternal and genital plates of *Entonyssus glasmacheri*, adapted from Vitzthum (1935), *Z. Parasitenk.* **7**, 709–16.
Fig. 7. Sternal and genital plates of *E. ewingi*, adapted from Hubbard (1938), *Amer. Midl. Nat.* **21**, 657–62.
Fig. 8. Sternal and genital plates of *E. vitzthumi*, adapted from Schmidt (1940), *J. Parasitol.* **26**, 309–13.
Fig. 9. Sternal and genital plates of *E. heterodontos* n.sp.
Fig. 10. Sternal and genital plates of species incertae sedis from *Pituophis sayi sayi*.
Fig. 11. Chelicerae of *O. natricis* n.sp. lateral view.
Fig. 12. Chelicerae of *O. elaphes* n.sp. lateral view.
Fig. 13. Chelicerae of *E. heterodontos* n.sp. mesial view.
Fig. 14. Chelicerae of *E. heterodontos* n.sp., ventro-mesial view.

Table 1

Classification	No. examined	Locality collected	Incidence of mites	
Family BOIDAE				
Charina bottae (Blainville)	3	Corvallis, Oregon	0	
Family COLUBRIDAE				
Diadophis amabilis occidentalis Blanchard	3	Corvallis, Oregon	0	
D. punctatus arnyi Kennicott	1	Atchison, Kansas	0	
Heterodon contortrix contortrix (L.)	1	Ottumwa, Iowa	29	*Entonyssus heterodontos* n.sp.
	1	Iowa City, Iowa	0	
Coluber constrictor flaviventris (L.)	1	Iowa City, Iowa	9	Species incertae sedis B
	1	Nebraska City, Nebraska	0	
	2	Ottumwa, Iowa	0	
	2	Memphis, Tenn.	0	
C. flagellum flavigularis (Hollowell)	1	Little Rock, Arkansas	0	
Opheodrys vernalis (Harlan)	2	Iowa City, Iowa	0	
Drymarchon corais couperi (Holbrook)	1	Eustis, Florida	0	
	11	Sarasota, Florida	0	
Elaphe guttata (L.)	1	Memphis, Tenn.	0	
E. obsoleta obsoleta (Say)	1	Iowa City, Iowa	4	*Ophiopneumicola elaphes* n.sp.
	1	Buffalo, New York	22	*Ophiopneumicola elaphes* n.sp.
	2	Memphis, Tenn.	0	
E. vulpina (Baird & Girard)	2	Iowa City, Iowa	0	
Pituophis catenifer annectens (B. & G.)	2	San Diego, California	0	
P. sayi sayi (Schlegel)	2	Iowa City, Iowa	0	
	3	Iowa City, Iowa	4, 7, 12	Species incertae sedis A
Lampropeltis calligaster (Harlan)	1	Ottumwa, Iowa	1	*Entonyssus heterodontos* n.sp.
L. getulus floridans Blanchard	1	Sarasota, Florida	0	
L. getulus holbrooki Stejneger	1	Little Rock, Arkansas	1	Species incertae sedis A
	1	Gramercy, Louisiana	0	
L. getulus californiae (Blainville)	1	San Diego, California	0	
L. triangulum syspila (Cope)	2	Iowa City, Iowa	0	
Contia tenuis (Baird & Girard)	1	Corvallis, Oregon	0	
Natrix cyclopion cyclopion (Dumeril & Bibron)	1	Gramercy, La.	0	
N. cyclopion floridana Goff	1	Sarasota, Florida	0	
	2	Ocala, Florida	0	
N. grahamii (Baird & Girard)	1	Gramercy, La.	0	
N. rhombifera rhombifera (Hallowell)	5	Little Rock, Arkansas	0	
	2	Dallas, Texas	0	
N. sipedon sipedon (L.)	1	Atchison, Kansas	7	*Ophiopneumicola natricis* n.sp.
	14	Iowa City, Iowa	0	
	3	DeKalb, Illinois	0	
N. sipedon confluens Blanchard	2	Gramercy, La.	0	
N. sipedon pictiventris Cope	3	Ocala, Florida	9, 11, 16	*Ophiopneumicola natricis* n.sp.
	4	Ocala, Florida	0	
	1	Eustis, Florida	0	
N. taxispilota (Holbrook)	1	Ocala, Florida	0	
Storeria dekayi (Holbrook)	1	Iowa City, Iowa	0	
Thamnophis radix (Baird & Girard)	5	Milford, Iowa	0	
	2	DeKalb, Illinois	0	
	7	Iowa City, Iowa	0	
T. sauritus proximus (Say)	2	Iowa City, Iowa	0	
T. sirtalis sirtalis (L.)	4	DeKalb, Illinois	0	
	16	Iowa City, Iowa	0	
T. sirtalis infernalis (Blainville)	3	Corvallis, Oregon	0	
Family CROTALIDAE				
Agkistrodon mokasen cupreus (Rafinesque)	2	Atchison, Kansas	0	
Sistrurus catenatus catenatus (Rafinesque)	1	Atchison, Kansas	0	
Crotalus horridus atricaudatus Latreille	5	Atchison, Kansas	0	

(1) Shape of sternal and genital plates (see Figs. 8, 10).

(2) This form shows a variation in respect to the angle at which the tip of the fixed chela is bent. In some of the specimens, the angle is about 90 degrees, as in *E. vitzthumi*, while in others the angle is about 135 degrees, as in *E. halli*.

(3) 'Ocelli' in pulvillus I are prominent in this form, whereas they are not figured or mentioned in the description of *vitzthumi*.

B. Nine mites were taken from the lung of a specimen of *Coluber constrictor flaviventris* (L.) from Iowa. These mites were taken from a snake which had been preserved in formalin for several months, and unfortunately were in a rather poor state of preservation. However, they greatly resemble *Ophiopneumicola colubri*, from which they differ in that: (1) Coxae I possess setae. (2) Pulvilli possess digitiform processes not shown in *O. colubri*. (3) Ultimate segment of palpus bears a furcate seta. (4) Genital plate is of different shape, and in some specimens bears a pair of setae.

Because of the great similarity of these forms to *Entonyssus vitzthumi* and *Ophiopneumicola colubri* respectively, it would seem desirable to defer an attempt at classification until further specimens have been secured for comparison.

DISCUSSION

On the basis of information lately received by the writer, the value of 'legs all similar' as a means of separating *Entonyssus* and *Ophiopneumicola*, appears to be nil. This character was included in the diagnosis of the genus *Entonyssus* by Ewing in 1922, and Hubbard (1938) mentions it as one of the characters distinguishing *Entonyssus* from the genus *Ophiopneumicola*, which he set up at that time. However, in all the species described for either genus since that date, tarsus I is cylindrical, and of equal diameter throughout its length, unlike the tarsi of the other legs.

Recently Dr H. E. Ewing kindly examined the type specimen of *Entonyssus halli*, which is the type species of the genus, and informed the writer that *E. halli* too had the cylindrical tarsus I, in common with all other species of the subfamily.

The writer is of the opinion that the records of *Lampropeltis calligaster*, *Lampropeltis g. holbrooki*, and *Natrix s. sipedon*, as hosts of *Entonyssus heterodontos*, species incertae sedis (from *Pituophis s. sayi*), and *Ophiopneumicola elaphes* respectively, are accidental, and that the above are not the usual hosts for these mites. In each case, the snake had been kept in a cage with infested snakes, which may be regarded as the normal hosts. In the first two instances, only one mite each was found, and the third species contained only seven mites. As the mites are viviparous, transmission must have been effected in the close quarters of the cages.

In each of the species of mites described for the subfamily Entonyssinae, the ventral plates, especially the sternal plate, are different from those of any other form, and therefore constitute a valuable means of identification. Illustrations of the sternal and genital plates of all species of Entonyssinae, except *Entonyssus halli*, and *E. rileyi*, are figured on Figs. 1–14. Illustrations were not included in the original descriptions of the two latter species, and Dr Ewing has informed the writer (personal communication) that the condition of the type specimens in the United States National Museum is such that it is impossible to trace the outline of the plates.

SUMMARY

1. Three new species of dermanyssid mites of the subfamily Entonyssinae are described. These are: *Entonyssus heterodontos* n.sp. from *Heterodon contortrix contortrix*, *Ophiopneumicola elaphes* n.sp. from *Elaphe obsoleta obsoleta*, *Ophiopneumicola natricis* n.sp. from *Natrix sipedon pictiventris*.

2. Keys for the identification of male and female mites of the Entonyssinae are given.

3. 141 snakes, representing thirty-five species, were examined for lung mites. Thirteen individuals, representing eight species, were found to be infested.

4. Two species incertae sedis, one from *Pituophis sayi*, the other from *Coluber constrictor flaviventris*, are discussed.

5. Problems of classification and distribution of this group of parasites are discussed.

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RESEARCHES ON THE INTESTINAL PROTOZOA OF MONKEYS AND MAN

XI. THE CYTOLOGY AND LIFE-HISTORY OF *ENDOLIMAX NANA*

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(With Plates I & II and 1 Text-figure)

Inest sua gratia parvis.
—Latin Proverb.

CONTENTS.—I. Introductory, p. 134. II. Methods, material, and preliminary data, pp. 135–40: (1) Cultivation, p. 135; (2) Strains studied, p. 137; (3) Viability of cysts, p. 138; (4) Cytological technique, p. 139. III. Descriptive, pp. 140–53: (1) Life-history in general, p. 141; (2) Cytology of trophic amoebae, p. 141; Notes on *Sphaerita*, p. 144; (3) Division, p. 145; (4) Precystic amoebae and encystation, p. 148; (5) Cysts, p. 148; Note on filamentar inclusions, p. 149; (6) Excystation, p. 150; (7) Metacystic development, p. 151. IV. Discussion, p. 153. Summary, p. 156. References, p. 157. Explanation of Plates I and II, p. 157.

I. INTRODUCTORY

In the fifth instalment of these *Researches* (1933) I gave a short account of the chief experimental work on which the present paper is based. Since then I have continued my studies and brought them to a stage which would have been satisfactorily complete—if not completely satisfactory—but for external circumstances over which I have no control. My work on *Endolimax nana* has been finished—as it was begun, in 1916—under difficulties imposed by war-time conditions in London. This will explain—though it may not excuse—some of its defects.

The present memoir is, as will be evident, a sequel to that published in 1933. In my monograph of 1919, moreover, I have given a full account of all that had been accurately ascertained about *E. nana* up to that date. I shall therefore assume that the expert reader is already acquainted with these two earlier publications, so that it is unnecessary to repeat here what has there been recorded. I find little to contradict or correct in these previous accounts. For the reader unfamiliar with the present subject, however, I may prefix a few particulars which will enable him to understand what follows.

Endolimax nana is the smallest of the amoebae living in the human intestine. It is therefore the most difficult to study accurately. Though its largest amoeboid stages are as big as some forms of *Entamoeba coli* and *E. histolytica*, its smallest are far less than the smallest human leucocyte; while its cysts are but little larger—and often even smaller—than a human red blood-corpuscle. To study such an organism properly therefore demands much patience and perseverance, while all the resources of modern cytological and optical technique are not superfluous aids.

The amoebae of this species live commonly in the large intestine of Man, and in monkeys of the genus *Macacus* [= *Macaca* = *Silenus*]. They may possibly live also in other hosts. They are not pathogenic, and feed upon the intestinal contents—not its tissues. In their human and simian hosts the amoebae reproduce by division, but details of the process are at present unknown. It is known, however, that after a period of multiplication they encyst, and escape from the body with the faeces in the form of minute quadrinucleate cysts. These are now familiar to protozoologists, but certain details of their formation from the active uninucleate amoebae are still undescribed. When the cysts are swallowed by a man or a macaque they give rise to a new infection with amoebae, as I have proved by experiment:* yet how they hatch, and turn again into the well-known uninucleate amoebae, is also still undetermined.

I have attempted to discover all the unknown stages of development by cultivating and studying the amoebae outside the body, by methods which have proved successful with other species. In this I have at last succeeded—more or less—and the following pages record my findings.

If any reader should think that my description is unnecessarily detailed, I would ask him to remember that the details alone have hitherto been lacking from our knowledge of this amoeba. Moreover, these details are all-important; for *E. nana* is the type of the genus *Endolimax*, and it is therefore essential that its characters should be established precisely. We cannot understand this organism and its relations to the other amoebae of the human bowel—and others living in other hosts—until this information has been acquired.

* See Dobell (1933).

A word may be added about the illustrations accompanying this paper. Some of the figures on the plates are very small: but so are the organisms which they depict, and it seems to me undesirable to represent them larger. They were all drawn at a uniform magnification (2000 diameters, as here reproduced), and are therefore exactly comparable with the figures of *E. histolytica* (1928), *E. coli* (1938), and *Dientamoeba* (1940) published in earlier instalments of this series. This magnification is not accidental, but was determined as that most suitable—for all species—when I began these studies. A higher magnification—3000, 4000, or even 5000 diameters, which some other workers have adopted—appears to me misleading. With the best optical apparatus a magnification of 1000 diameters is adequate to reveal every resolvable detail to a normal human eye. Greater enlargement—especially if drawings are made in line and stipple—introduces errors, and gives a false impression of detail which is really invisible (or incapable of resolution) in the actual specimens. The uniform monochrome of the reproductions cannot of course represent the true colours of the originals, which are of various tints: otherwise my figures have been drawn as accurately as possible, and are in no way diagrammatic. I recommend them to the reader's special attention, as they are, I think, perhaps more objective records of my observations than my words. To me it has always been easier to draw correctly than to write correctly.

For the delay in publication I am not wholly to blame. Circumstances, as already indicated, have been unfavourable. Nevertheless, I am responsible to some extent because I have hesitated to make my first findings known before I could confirm them properly. To my critics I would therefore venture to say, in the words of the first and greatest of all protozoologists: 'In these observations I have spent a lot more time than many people would believe; yet I made them with pleasure, and paid no attention to those who say to me "Why take so much pains?" and "What's the use of it?": because I don't write for such folks, but only for Philosophers.'*

II. METHODS, MATERIAL, AND PRELIMINARY DATA

The methods which I have used for studying the life-history of *E. nana* are essentially the same as those previously employed for *E. histolytica* and *E. coli*.† It is therefore unnecessary to describe them again. The material used and methods of cultivating *E. nana* have also been partly described elsewhere.* But additional data have been acquired by further experience, and it will be convenient to record these here before giving an account of the life-history in detail. Accordingly, the following sections (1–4) are to be regarded as supplements to what has already been published, though I shall repeat a few particulars in order to make the subject intelligible to readers unacquainted with my earlier work.

(1) *Cultivation*

Endolimax nana can be readily cultivated *in vitro* by methods previously described.† It grows well in all the media of Boeck-Drbohlav type which I usually employ. For the reader's convenience I may note these here, with the abbreviations by which I designate them:

(i) Coagulated whole egg (diluted with Ringer's fluid) covered with horse-serum (diluted with the same fluid, 1 : 8) = **Ehs**.

(ii) The same solid covered with dilute egg-white (in Ringer's fluid) = **Ere**.

(iii) Inspissated horse-serum covered with Ringer-eggwhite = **HSre**.

(iv) The same, but covered with dilute horse-serum (1 : 8) = **HShs**.

Pure strains of *E. nana*—i.e. those containing this species of amoeba only, free from all other intestinal protozoa and *Blastocystis*, but accompanied by bacteria suitable for its growth—usually grow freely at first in these four media; but on continued cultivation it is usually found that some are better than others, and occasionally one or other ultimately proves to be unsuitable. This is probably determined by the bacterial flora, which is different in every cultivated strain. In my experience almost all strains can be easily cultivated continuously in Ehs, though it is unusual to find one which can be maintained indefinitely in HSre. I may add that I have now studied over a hundred strains derived from Man, *Macacus sinicus*, *M. rhesus*, and a man (myself) experimentally infected with a *sinicus*-strain.

Addition of solid rice-starch to the culture-medium does not generally favour the growth of the amoebae. Most strains will ingest it readily, but I have had a few which refused to eat starch in any form. No strain yet studied—and I have carefully tested a large number—has ever been found to ingest human red blood-corpuscles. As a rule *E. nana* feeds on bacteria of all sizes, from large (*B. coli*, etc.) down to the smallest organisms present; but it appears to prefer small and very small species. (Many of these are unidentified anaerobes.) It also, at times, takes up bacterial spores in large numbers.

* Leeuwenhoek, *Send-brief*, II, p. 22. [Translated.] Letter dated 17 Dec. 1712. Published 1718.

† See Dobell (1928, 1931, 1936, 1938).

* See Dobell (1933).

† See Dobell & Laidlaw (1926), Dobell (1933).

Though it is easy to obtain rich cultures of *E. nana* in the amoeboid form, and to propagate them *ad libitum*, it is far from easy to obtain strains which will encyst readily and regularly. These, however, are essential for a study of the whole life-history, and I have spent much time and labour in attempting to secure them. One such strain (Σ., from *M. sinicus*) I have already described.* This grew excellently in Ehs medium, and for a time formed abundant cysts—usually on the fourth day of incubation. These cysts, if removed from the incubator and kept at room-temperature (for any time up to 2–3 weeks), hatched readily when inoculated into fresh medium and incubated at 37° C. After some weeks, however, this strain gradually ceased to form cysts and was therefore abandoned.

Most strains of *E. nana* which I have hitherto isolated from human beings or macaques have been, from the beginning, non-encysting strains. They never produced any cysts at all, and all my attempts to induce them to do so have failed. But a few have behaved like Strain Σ., and after forming cysts more or less abundantly, for a variable number of generations, have ceased to do so. Some of these strains, moreover, have produced cysts which appeared normal in every way but which refused to hatch on incubation in any medium.

In 1937 I obtained an excellent human strain (*LO.*) which encysted freely in Ehs medium from the very beginning. It also grew well in other media, but in these it would not encyst. It differed from Strain Σ. in that the cysts appeared in cultures usually after incubation for only 2 days (or occasionally 3). The cysts hatched also in the same medium, but usually much better in HSre. Addition of small amounts of meat-extract or broth to the liquid often appeared to favour excystation. When this strain (*LO.*) was cultivated continuously in the same way as Strain Σ.—i.e. by inoculating subcultures every 2 or 3 days (when many amoebae had encysted)—it soon ceased to form cysts likewise, and became a non-encysting strain. I found by experiment, however, that I could propagate it continuously as an encysting strain by making subcultures every day (i.e. before encystation occurred). These subcultures produced good crops of cysts if incubated further for 2–3 days; and the cysts so formed behaved normally, and hatched readily when inoculated into fresh medium. I kept this strain going in this way for many months, and was able to study all stages of the life-history *in vitro*. Before I had completed this study to my own satisfaction, however, the strain underwent an unexpected change. It continued to form cysts abundantly, but these gradually became more and more difficult to hatch. On incubation, an increasing percentage degenerated and died; and finally I could not make them hatch at all. As the strain thus became useless for further study of the metacystic stages, I had to abandon it.

After I had succeeded in studying all stages in the life-history (more or less completely) in a simian and in a human strain of *E. nana*—and had made very many confirmatory though incomplete observations on numerous other strains from macaques and men—I desired, in order to round off the work, to make a similar study of a simian strain from Man. The only available subject was myself—experimentally infected with Strain Σ. in 1925.* Since that date I have made innumerable attempts to isolate a really good encysting strain from my own stools, but nearly all have failed. I have carefully isolated and studied over 50 such strains, but most of them have refused to encyst. A few have formed cysts for a few days, or even a few weeks, after isolation: they have then ceased to do so, under many different and varied conditions. A few strains, moreover, have formed cysts in some abundance—when first isolated—but these have hatched badly or not at all. Most strains have been readily propagable in the amoeboid stage, but useless for study of precystic and metacystic development. This applies to strains cultivated from the most diverse original material—normal formed stools, soft or loose stools, and liquid stools obtained by saline purgatives. The initial material has contained cysts or amoebae only, or mixtures of both in various proportions. All proved equally disappointing.

I have at last succeeded, however, in obtaining from myself a satisfactory encysting strain (*SD.*) in which I have been able to induce both encystation and excystation fairly consistently for over 2 years. It differs from all others in developing best in HShs medium. It will also grow freely in Ehs and HSre continuously; but it will not encyst continuously in either of these media. In HShs, on the other hand, it encysts excellently (when grown in the manner described for Strain *LO.*, but not otherwise): and the cysts will hatch in HShs, HSre, and Ehs media —the best hatches being usually obtained in the first and the worst in the last. I have now kept a number of lines going by cultivating the amoebae by daily transplantation for a few days or weeks; then causing them to encyst; then keeping the cysts for several days or weeks; and finally hatching them again, and repeating the process. Several lines propagated in this way have now encysted and hatched over 30 times in succession.

I have used this strain (*SD.*) to confirm my previous observations, and for various other purposes. It has been of great use, but has never been entirely satisfactory. From time to time it has failed to encyst, or has produced crops of cysts which hatched

* See Dobell & Laidlaw (1926), Dobell (1933).

* See Dobell (1933).

badly or not at all—despite all my efforts to keep all cultural conditions constant. I cannot explain all my failures in this respect, but some were probably due to the unavoidable variability of the culture-media employed. Eggs and serum are not pure chemicals, and media prepared from them—with every care, and by identical methods—therefore vary in composition from batch to batch. At present it is impossible to define or eliminate such elusive variations.

The factors which determine encystation and excystation in *E. nana* are still obscure. All my attempts to define them precisely—and they have been many—have hitherto failed. Two of my best strains, which originally completed their life-cycle *in vitro*, had to be abandoned because they changed their habits on continued cultivation under conditions kept as constant and stable as possible. As already noted, the first (Σ.) ceased to form cysts, while the second (*LO.*) continued to encyst but the cysts would no longer hatch.

I have no doubt that in *E. nana* both encystation and excystation are, to a large extent, determined by the concomitant bacterial flora—just as they are in *E. histolytica* and *E. coli*.* In both these species it is not difficult to prove that certain bacteria are necessary for cyst-formation and also for the hatching of the cysts. By adding the bacteria from an encysting strain to a non-encysting strain, for example, encystation can always be produced. Yet with *E. nana* this procedure seldom succeeds. I have made scores of experiments in this connexion, and nearly all of them have failed. It is usually impossible, in my experience, to cause encystation in a non-encysting strain by growing it with the bacteria from an encysting strain. After 18 years' work and experiment with *E. nana*, I am still unable to obtain hatchable cysts of this species at will, as I always can with *E. histolytica* and *E. coli*. I have thus had to depend largely on luck for most of my good material.

(2) *Strains studied*

In the course of the present work I have cultivated—as just noted—a large number of strains of *E. nana*, though only a few of these have proved suitable for detailed study of the life-history. I give a brief synopsis of the relevant data, for the chief strains here studied, in Table 1—followed by a few explanatory particulars.

Table 1. *Strains of* E. nana *specially studied in the present researches*

Strain	Host of origin	Period of cultivation	No. of serial subcultures
Σ.	*M. sinicus*, natural infection	21. vii. 25–31. i. 26 = 6 months	51
Δ3.	Man, experimentally infected with Strain Σ.	24. ix. 25–30. x. 25 = 5 weeks	20
ΔX.	Ditto	28. ii. 40–27. v. 40 = 3 months	50
dx.	Ditto	5. vi. 40–1. viii. 40 = 2 months	28
SD.	Ditto	7. viii. 40–13. x. 42 = 2¼ years	133
RN.	*M. rhesus*, natural infection	19. iv. 35–31. v. 35 = 6 weeks	20
LO.	Man, natural infection	24. v. 37–14. vi. 38 = 13 months	98

Notes on Table 1.—Strain Σ. has already been described in some detail (1933). It was obtained from my tame monkey Susanna, from whom—and from other macaques of the same species—I have isolated and studied many other strains at various other dates. It was the only good *encysting* strain ever secured from such sources, and grew excellently in all the usual media.

Strains Δ3., *ΔX.*, *dx.*, *SD.* are only a few of the numerous strains (over 50) which I have isolated from myself since my experimental infection with Strain Σ. in 1925 (see Dobell, 1933). These 4 are specially mentioned because they were used for experiments or observations here described, while 3 of them furnished material for accompanying illustrations. All grew well in the usual media, but *SD.* was the only one which encysted regularly and freely.

Strain *RN.* was the best of many studied from several individuals of *M. rhesus*, and was derived from my tame monkey Rosa. It grew excellently in all media, but never encysted *in vitro*.

Strain *LO.*, studied in great detail, is my only human strain which has encysted freely *in vitro*. It was derived from the normal stool of a patient—a British lady who had lived in Malaya, and had never suffered from dysentery—whose physician sent it to me to examine for a suspected infection with *E. histolytica*. This parasite was not found; but in addition to *E. nana* I discovered *Dientamoeba fragilis* in the original specimen. (This species was easily eliminated, and not cultivated further.)

Besides the intestinal strains noted in Table 1, I have studied a number derived from the vaginal contents of *M. sinicus* [=*radiatus*] and *M. rhesus* (see Dobell, 1933, p. 439). But as all these proved to be non-encysting strains, and were not used in the present investigations, they are not included here.

It should be emphasized that all the foregoing strains were *pure*—that is, free from all other amoebae (and other protozoa and *Blastocystis*) but accompanied by stable mixtures of various species of bacteria favourable for their growth. They were all propagable *ad libitum* in the amoeboid stage, and were abandoned merely because they had served their purpose.

* Cf. Dobell & Laidlaw (1926), Dobell (1927, 1928, 1931, 1936, etc.).

(3) *Viability of cysts*

It is of practical importance to know how long the cysts of *E. nana*—and other intestinal amoebae—are able to remain alive. It is necessary to know, for example, how long they remain infective after leaving the body; and for experimental purposes we want to know how long they can be kept alive and capable of hatching to produce new cultures of amoebae.

The viability of cysts can, of course, be determined only under carefully controlled and defined conditions; and consequently culture-cysts alone are suitable for experiment. With cysts derived from faeces no really accurate tests can be made, as they are inevitably associated with unknown mixtures of bacteria and other things.*

I have already published (1933) the results of various experiments made with Strain Σ., which showed that cysts of *E. nana* are able to live at ordinary room-temperatures for at least 3 weeks. Later work has proved that they can survive for considerably longer periods at lower temperatures (10°, 5°, 2° C.). When I obtained another good encysting strain (*LO.*) I attempted to determine their viability with greater precision.

A first series of experiments was made with cysts of Strain *LO.* kept in an ice-chest at approximately 10° C. I found that they would hatch (when incubated at 37° C. in fresh medium) fairly regularly for 3 weeks (22 days), but afterwards gave very irregular results. Most cysts failed to hatch after the third week, but I obtained positive cultures occasionally up to the 36th day. This proved that the cysts could sometimes live for as long as 5 weeks, though most of them died earlier.

As the results of about 40 experiments performed in this way were inconclusive, I resolved to repeat them under more carefully controlled conditions. A second series of experiments was therefore made with cysts kept at a lower and more constant temperature (5° C.). Preliminary results showed regular hatching up to 18 days; and I then obtained positive cultures at 41 and 45 days, though most cysts tested between the third and sixth week were found to be dead. A third series of experiments, however, consisting of nearly 50 cultures made on the 20th to the 67th day gave entirely negative results: and I then found that the cysts of this strain had lost their power of hatching altogether.† Even those only a few days old hatched very badly or not at all. I could therefore make no further tests with them, and had to abandon the experiments temporarily. They proved, nevertheless, that cysts of *E. nana* may live as long as 45 days at 5° C.

When I obtained, at last, another good encysting strain (*SD.*), I made another attempt to determine the viability of its cysts; and in spite of considerable difficulties and many interruptions, I have now collected sufficient data—during more than 2 years—to fix an end-point with greater precision. In these experiments, moreover, I was able to control the temperature more accurately (by the use of better apparatus). The cysts, when removed from the incubator, were cooled to room-temperature, and after a few hours placed in a refrigerator kept at a constant temperature of 2° C. Here they were left for various periods, and then tested by inoculation into fresh medium and incubation at 37° C. A different batch of cysts was used for each test, with very few exceptions, and in the tests of older cysts a large sample was usually incubated. Medium proved to be favourable for hatching was alone used. As a rule, no culture was regarded as negative unless it had been incubated for 5 days; and whenever there was any doubt of the result, subcultures were made and carefully examined.

The final results of this series of tests are shown in Table 2. The signs + and − each indicate a positive or negative culture (entered in the order in which the cultures were made). The entry 0 means that no test was made for the day in question. The experiments are summed-up for 5-day periods. It will be seen that more tests were made during the earlier period, but this is merely because cysts were more often hatched at these times for the purpose of other experiments and for continuing the strain.

The comparatively small number of tests made after the 38th day is due to the circumstance that cysts above this age were not often available for study. All material was carefully controlled microscopically; but after keeping cyst-containing cultures for more than about 5 weeks I frequently found that all the cysts had degenerated and died. They were so obviously dead—sometimes, indeed, having all disintegrated—that it appeared a waste of time and culture-medium to incubate their remains. It is undoubtedly exceptional for cysts to live more than 40 days at 2° C., but many observations which I have made corroborating this statement are not included in the table.*

Table 2, which summarizes 220 experiments, shows that cysts of *E. nana* (Strain *SD.*) regularly live for 25 days at 2° C., but later die off in increasing numbers up to the end of the second

* I have said this before, but it seems necessary to emphasize it as people are still publishing 'experiments' made by methods which can obviously yield only fallacious results.

† See p. 136 *supra*.

* No cysts were tested before the 3rd day, because I have found by experiment that accompanying amoebae can occasionally survive at low temperatures for as long as 48 hours. Cf. Dobell (1933).

Table 2. *Tests of viability of cysts* (E. nana, *Strain* SD.)

Age of cysts days	Results of cultures		Age of cysts days	Results of cultures	
1	0		31	+ − + −	
2	0		32	− − − + + +	
3	+ +	16+	33	− +	10+, 11−
4	+ + + + + + +		34	− + − +	
5	+ + + + + + +		35	− − + − +	
6	+ + + + +		36	− + + − +	
7	+ + + + + + + + + +		37	+ + − +	
8	+ + + + + + + +	42+	38	+ − − + − − − −	8+, 12−
9	+ + + + + + + + +		39	− −	
10	+ + + + + + + + + +		40	−	
11	+ + + + + + + + + +		41	−	
12	+ + + + +		42	−	
13	+ + + + + + + +	37+	43	+	1+, 5
14	+ + + + + + +		44	− −	
15	+ + + + + + +		45	−	
16	+ + + +		46	− − − + −	
17	+ + +		47	−	
18	+ + +	22+	48	−	1+, 8−
19	+ + + + + +		49	−	
20	+ + + + + +		50	−	
21	+ + + + − + + +		51	−	
22	+		52	−	
23	+ +	19+, 1−	53	+	1+, 4−
24	+ + + + + +		54	−	
25	+ + +		55	−	
26	−		56	−	
27	− + +		57	−	
28	+ + − +	11+, 4−	58	−	1+, 6−
29	+ +		59	+	
30	+ − + + +		60	− − −	

month. The maximum time for which any cysts have survived is 59 days.

I have thought it desirable to publish these exact figures for at least one strain, as it seems unlikely that any other worker has yet had the material and patience to study this subject in such detail.

Although, as already noted, it is not possible to make any similarly exact experiments with cysts passed in the faeces, I may add that I have also collected many data about these. I have found, in general, that natural cysts seldom live for more than a fortnight in faeces kept at ordinary room-temperatures, though they may live longer in cold water or Ringer's fluid. The maximum time of survival—as determined by cultivation—appears to be about 3 weeks; but on one occasion I have obtained a positive culture from cysts kept in water on my bench for 26 days. The cysts were derived from a macaque (*M. sinicus*)—not a human being—and at present this constitutes my record for such cysts. It seems reasonable to conclude that, in nature, the cysts of *E. nana* after leaving the body seldom remain alive and infective for more than 2 or 3 weeks in temperate climates. In tropical temperatures they must perish much more rapidly.

(4) *Cytological technique*

I add a few notes here on the cytological technique used in the present researches, as the subject is of fundamental importance. Every cytologist prefers his own methods, of course, and in this respect I am not peculiar. In general, therefore, I have used the same methods as those which I have already described for *E. histolytica* and other intestinal amoebae.* It will be unnecessary to repeat the cautions and precautions previously emphasized.

E. nana is so small, and so hard to study at many stages, that I have had to spend a very long time in working out certain details of its development. Its nucleus, and nuclear division, are peculiar—indeed unique among known amoebae; and I have therefore devoted special attention to them. For these studies I have found ordinary smears unsuitable, and have therefore devised a method of making films which has enabled me to obtain much better

* See Dobell (1928), etc.

preparations. This I have described elsewhere,* and the account of nuclear division here given is based upon preparations made by this method.

All good methods of fixation and staining give good results with *E. nana*. For fixation I prefer Schaudinn's fluid (sublimate-alcohol) with 5% glacial acetic acid added, and Bouin's fluid (original aqueous formula). I have also used successfully various mixtures of sublimate and osmium tetroxide, and of sublimate and formol. Flemming's fluid has generally given less satisfactory results, and has been used only for special purposes. Particularly good fixation is obtainable with a modified form of Zenker's fluid, which I have used frequently: and as this is also an excellent fixative for other amoebae—and for general purposes—I give the formula here:

Saturated aqueous solution of corrosive sublimate ($HgCl_2$) ...	50 c.c.
5% aqueous solution of potassium bichromate ($K_2Cr_2O_7$)	50 c.c.
Glacial acetic acid	5 c.c.

The best staining methods, for the study of nuclear details, I have found to be Heidenhain's iron-alum haematoxylin and my alcoholic iron-alum haematein,† Mallory's phosphotungstic haematoxylin, and my tungstic and molybdic haematoxylin stains.‡ I have found borax-carmine useful for studying 'chromatin' elements, and Best's carmine excellent for demonstrating glycogen in cysts.§ Chlorazol black‖ is also very useful for glycogen, and gives clear pictures of nuclear details and cyst walls in addition.

As certain workers on amoebae have attached particular importance to the results obtained with special mixtures or combinations of stains, I must add that I have used most of these also—especially Mann's methylblue-eosin, the various Romanovsky stains (Giemsa, Leishman, etc.), and safranin-light-green. All these give very pretty preparations when properly employed. I cannot say, however, that they have rendered me any special service. Results obtained by these methods will be noted later. But it should be noted here that I employ Mann's stain in the manner previously described—differentiating in a very dilute alcoholic solution of orange G (not in alkali):* Romanovsky stains I employ by wet methods throughout (using my own well-tried technique). I cannot agree with those authors who maintain that drying after staining has no deleterious effects.†

Feulgen's stain has proved of little utility. I hoped to obtain valuable information from its use, but my hopes have not been fulfilled. The 'nuclear reaction' in *E. nana*, at all stages of development, has invariably proved negative in my hands. This matter will be considered later in more detail,‡ but I may add here that my failures have not been due to inexperience or technical errors. I have used this method in many other cases with complete success, and am well acquainted with its principles and practice. The dyes which I have used have all been tested and controlled on other objects, with which they gave positive results. In preparing the staining solutions I have used both sodium sulphite (Na_2SO_3—which appears to be the 'anhydrous sodium bisulphite' prescribed by Feulgen) and potassium metabisulphite ($K_2S_2O_5$). Both have yielded excellent and almost colourless solutions with good samples of 'basic fuchsine' (magenta, rosaniline hydrochloride, etc.). After staining, I never omit washing with SO_2 solution—properly prepared. As a rule I use light-green or orange G as counterstain. I mention these points in order to forestall criticisms such as those which have been levelled—and not without justice—against some other workers who have previously obtained 'positive' or 'negative' Feulgen reactions with amoebae.§

III. DESCRIPTIVE

In this section I shall give an account of the whole life-history of *E. nana* as I have observed it in cultures grown and studied under optimal conditions. By 'optimal' I mean those which, on repeated trial, with every factor controlled and stabilized as far as possible, have given me constant and uniform results.

I have every reason to believe that the development which I describe is that which normally occurs in the human body, though at present this cannot be proved. Nevertheless, it is certain that the trophic amoebae and cysts develop *in vitro* exactly as they do in the body: and there is thus no reason to suppose that the other stages—which at present can be studied only in cultures—do not likewise correspond.

The development of *E. nana* in Man, so far as it is known, has already been described;‖ and I

* See Dobell (1942). † See Dobell (1914).
‡ See Dobell (1942). § See Dobell (1919).
‖ See Cannon (1937, 1941). I called attention to the fact that this stain can be used to demonstrate glycogen in a previous paper (1938): but I have since found that all samples of the dye are not suitable for the purpose. The sample which I originally used—sent to me by Prof. Cannon in 1936—gave (and still gives) excellent results; but another sample, obtained from British Drug Houses Ltd., has proved to be almost useless. The matter evidently requires further investigation.

* See Dobell (1919), p. 7.
† Cf. Wasielewski & Kühn (1914).
‡ See pp. 143, 147, 154 *infra*.
§ Cf. Bogdanowicz (1930).
‖ See Dobell (1919), pp. 104–8.

shall assume that the reader is acquainted with this description—to which I shall have frequent occasion to refer, though I cannot repeat it here. The present account, however, is complete in itself.

(1) *Life-history in general*

Before proceeding further it will be convenient to give a brief outline of the whole life-history, and recall the chief terms which I use in describing the various stages.

In general, *Endolimax nana* develops in the same way as *Entamoeba histolytica** and *E. coli*,† though there are important differences in detail. The ordinary uninucleate amoeboid forms, which I call *trophic* amoebae, live an active life and multiply by fission (bipartition, with mitotic division of the nucleus). After a time, they produce peculiar *precystic* amoebae which undergo *encystation*. The resultant *cysts*—which are multinucleate (typically 4-nucleate) when mature—are resistant forms capable of survival in a resting state outside the body (or the culture-tube). When ingested (or inoculated into fresh culture-medium and incubated at body temperature) they hatch, or undergo *excystation*, and liberate their contents. Each cyst discharges a single multinucleate (typically 4-nucleate) amoeba, which later divides into small uninucleate *amoebulae*; and these then grow up into trophic forms once more. The stages between hatching and the formation of trophic forms I call *metacystic*.

Although I have obtained the complete development *in vitro* of many different strains, I have not been able to study all in equal detail. My most complete series of division-stages in the trophic forms is derived from Strain *SD.*; and of encystation, excystation, and metacystic stages from Strain *LO*. These strains have therefore been illustrated and specially described for these stages respectively. It must be noted, however, that all strains—so far as they have been studied—appear to develop in the same way, so that descriptions of any one apply to any other. At all events, I have been unable to detect any significant difference in size, form, or cytological detail between Strain *LO*. and Strain *SD*. at any stage of development.

(2) *Cytology of trophic amoebae*

The trophic forms of *E. nana* are already so well known that complete redescription is unnecessary. But the most characteristic feature of this species is its nucleus, about which more must be said; for it can be studied more accurately in cultivated amoebae than in those—usually more or less degenerate—which are discharged with the faeces of men and macaques.

Well-grown trophic amoebae measure about 8–10 μ in diameter when rounded, though they may attain a much greater length (up to about 30 μ) when extended during active locomotion. The single nucleus is vesicular and spherical, measuring about 2–2·5 μ in diameter. A couple of typical individuals are depicted in figs. 1 and 2 (Plate I).* The nucleus at once attracts attention, owing to its unusual appearance: for it is entirely different from that of the common small free-living amoebae (miscalled '*limax* amoebae') with which *E. nana* is often compared. These possess a large spherical and central karyosome, easily visible in the living organism; whereas the karyosome of *E. nana* is polymorphic, and difficult to distinguish during life.†

To say that the karyosome in *E. nana* is polymorphic conveys no idea of the multifarious forms which it may assume. It is almost impossible to express the appearance of this structure in words, for hardly any two amoebae appear exactly alike. I have tried to show some of the variations in an earlier publication,‡ but must now try again—as I am now dealing with amoebae in cultures (not faeces), in which the polymorphism is even more conspicuous. Text-fig. A is an attempt to illustrate some of the commoner forms of karyosome seen in well-fixed and stained specimens. The amoebae are here outlined with the *camera lucida*, and their nuclei accurately filled in. (All the organisms shown are from the same preparation—a film made by the coverglass technique described previously.§ Strain *SD*. Fixation: sublimate-alcohol with 5% acetic acid. Staining: phosphotungstic haematoxylin. Magnification: 2000 diameters.)

In its simplest form—from which all the others may be derived—the karyosome appears as a large homogeneous globular mass of deeply staining material ('chromatin') lying at the centre of the nucleus (Text-fig. A, 1). It is separated from the

* See Dobell (1928).

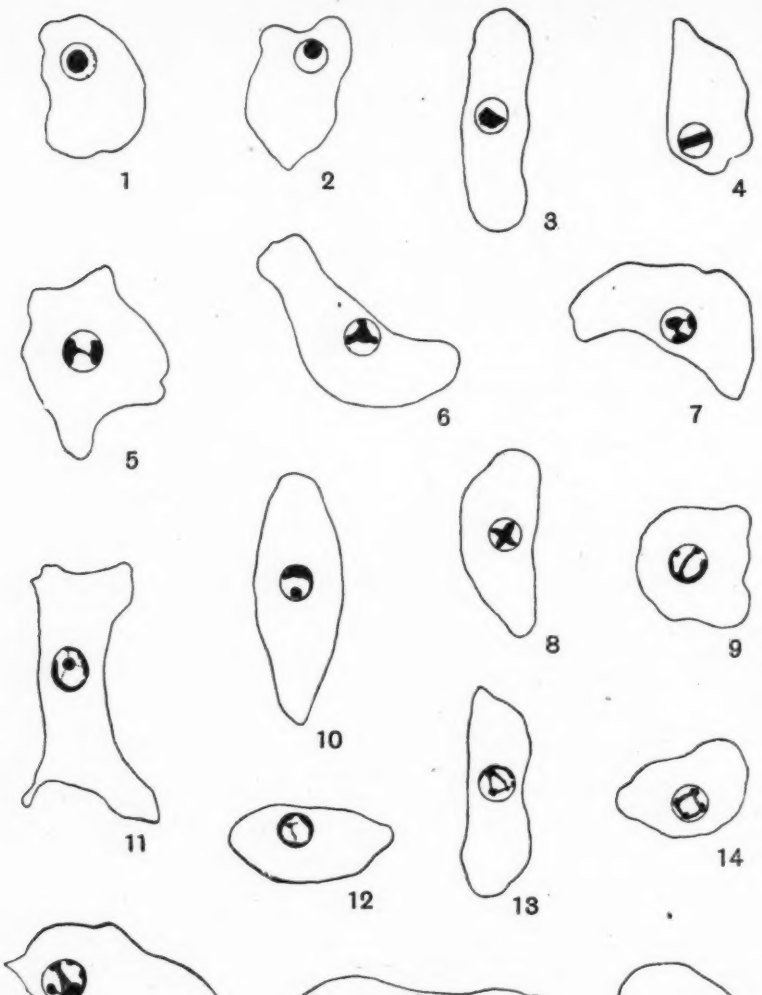
† See Dobell (1938).

* Other specimens (from human faeces) are shown in Dobell (1919), Pl. II, figs. 18–23. Smaller pictures, showing forms commonly seen during active movement, will be found in Dobell (1942), fig. E, p. 112.

† I shall continue to call the 'chromatinic' body in the nucleus of *E. nana* (and the '*limax* amoebae') a 'karyosome', though I am aware of the valid objections raised against this term. Some authors prefer to call it an 'endosome' (Minchin, 1912, pp. viii, 73) or, in German, '*Binnenkörper*': but it is surely evident that these supposedly non-committal words are equally objectionable, since they have similar implications if used generally. So long as one remembers that all the structures called 'karyosomes' are not homologous, in all protozoa, I see no real objection to this convenient and well-established word. But naturally I do not imply thereby that the 'karyosome' of *Endolimax* is identical with that of *Aggregata*, or the 'net-knots' of metazoan nuclei.

‡ See Dobell (1919), Pl. II, figs. 18–24.

§ See Dobell (1942).



Text-fig. A

well-developed nuclear membrane by a broad 'clear zone' in which no structure is usually visible. The nuclear membrane, however, is generally studded irregularly with a few minute beads—colourable with various dyes, but not giving the usual reactions of 'chromatin'. In fixed specimens these granules are often joined to the karyosome by fine radial 'linin' threads—perhaps products of fixation. Very commonly the karyosome itself is not central but eccentric (A, 2), and in contact with the membrane.

Moreover, the karyosome is frequently not spherical but square or polygonal (A, 3) in outline, and sometimes appears as a block or bar extending across the nucleus (A, 4). It may also be divided into two (A, 5), three (A, 7), or more, lobes or masses—connected by strands—or be drawn out

into three, four, or more processes so that it has the appearance of a triangle or a cross (A, 6, 8). Very often it appears to flow round the nuclear membrane—forming a crescentic mass at one pole, connected with a globule at the opposite pole (A, 10. See also Pl. I, fig. 2).

The karyosome may even flow round the inside of the nuclear membrane to such an extent that it gives the nucleus the appearance of a ring (in optical section) connected by fine strands to an eccentric granule or globule of variable size (A, 11, 12).* In its more complicated forms it appears as a system of threads and globules assuming the most diversified shapes (A, 9, 13, 14, 15, 16, 17). The figures will convey a better idea of the appearances than pages of description.

All these remarkable forms of the karyosome are to be seen in well-fixed and stained normal amoebae. They are certainly not artifacts due to faulty fixation. With all good fixatives the appearances are the same: and the fresher the material, and the more rapidly it is fixed, the greater is the variety of forms observable. Degenerate amoebae, or those subjected to cooling before fixation, frequently all show the karyosome as a rounded undifferentiated mass: but this is indeed an artifact, and does not represent its true form. It is the form commonly seen in the degenerate, dying, or dead organisms evacuated in human stools—upon which many accounts of the nucleus of *E. nana* have been erroneously founded.

The variable forms of the karyosome in *E. nana* suggest irresistibly that this structure is an unstable mass of fluid colloidal material, constantly undergoing changes of shape within the confines of the nuclear membrane. It is itself 'amoeboid'. I believe, indeed, that I have been able to witness its transformations in the living organism; but such observations are so difficult to make—owing to the active movements of normal amoebae, and the intervention of optical sources of error—that I do not ask others to accept what I have seen. I would only emphasize the fact that all the forms of karyosome just described are always visible in well-fixed and stained amoebae, and are not produced by faulty cytological technique.

I must also emphasize another point. The remarkable forms of the karyosome just noted are in no way related to the phenomena of division. They all occur in 'resting' nuclei—during the interdivision period—in active trophic amoebae. Appearances such as those illustrated in Text-fig. A, 5 may suggest division-stages, when first seen: but they have no relation to the mitotic figures which I shall presently describe. Of this I am now confident, after prolonged study of abundant material; but whether the various forms of karyosome succeed one another in any definite and constant order, during the interkinetic period, I am unable to determine. They can be arbitrarily arranged in various sequences—as will be evident; but that any of these represents an orderly series of stages through which the karyosome normally passes, it is impossible to state with certainty. My own impression is that there is no such definite sequence—the karyosome probably undergoing continuous changes of shape during life, like a drop of oil suspended in a watery medium. Its many forms could probably be satisfactorily accounted for, in terms of surface tension, if we knew its chemical composition and that of the liquid in which it lies.

The karyosome of *E. nana* is composed of 'chromatin'—using the term in its ordinary old-fashioned sense. It stains intensely with all 'basic' dyes—haematoxylin of all sorts (with iron alum, potash alum, phosphotungstic acid, ammonium molybdate, and other usual mordants), borax carmine, iron-chlorocarmine, chlorazol black, safranin, magenta, methyl green, methylene blue, etc. With Mann's stain (methylblue-eosin) it is coloured a bright cherry-red, and with the Romanovsky stains an intense reddish purple. But it gives no 'nucleal reaction' with Feulgen's stain, and is therefore not composed of 'chromatin' or 'euchromatin' according to modern nomenclature. The entire 'resting' nucleus—karyosome, clear zone, and nuclear membrane, with its granules—remains uncoloured when treated by the Feulgen technique.*

As several other workers have also failed to obtain positive results with the nuclei of other amoebae, and this has been attributed to faulty methods of hydrolysis or staining, it seems advisable to add the following particulars:

It has been claimed that positive results can be obtained with amoebae by prolonging the time of hydrolysis or staining beyond that usually sufficient for other objects. As a general practice I adopt the standard times of 4–6 minutes for hydrolysis (in $N/_1$ HCl at 60° C.) and 3–4 hours for staining in the reduced fuchsine solution. As these gave negative results with *E. nana*, I tried hydrolysing the films for various times up to 20 minutes, and staining for as long as 20 hours. My latest preparations were made with great care, and I have summarized the details of procedure in Table 3, which shows the results of my last 16 experiments. It is possible that by varying the times or methods of hydrolysis or staining positive results can be obtained, but my experience has been so uniformly negative that I have felt discouraged from continuing such attempts. It should be added that 2 different samples of basic fuchsine were used for preparing the stains, which were made up in 4 different ways (with different amounts of

* Nuclei of this form superficially resemble those of *Entamoeba* (e.g. *E. histolytica* or *E. coli*): but their structure is really very different, and should not deceive a careful observer. Cf. p. 154 *infra*.

* Cf. p. 140 *supra*.

Na_2SO_3 or $K_2S_2O_5$ and HCl). All these stains gave excellent positive results with other objects. The tests recorded in the table were made with 7 different strains of *E. nana*.

Table 3. *Feulgen reaction in* E. nana (*'resting' nuclei*)

Preparation no.	Duration of Hydrolysis min.	Duration of Staining hr.	Result
1954	4	4	Negative
1958	4	19	,,
1929	5	3	,,
2174	5	3¼	,,
1955	5	4	,,
1959	5	19	,,
1972	10	4¼	,,
2063	10	17	,,
2107	10	18	,,
2052	13	3½	,,
1974	13	4	,,
1973	15	3	,,
2051	15	3½	,,
2108	15	20	,,
2053	18	3½	,,
2058	20	4	,,

All the preparations listed in Table 3 were fixed with Schaudinn's fluid (sublimate-alcohol, with and without acetic acid). Feulgen himself originally stated that oxidizing agents—such as osmium tetroxide and chromic acid—should not be used as fixatives in his technique; but later workers have obtained excellent results after fixation in Flemming's fluid and other combinations containing 'osmic acid'. I have confirmed these findings with many objects, but not with *E. nana*. With this organism, however fixed, my results have invariably been completely negative.

A few years ago I discovered that some old material (*Aggregata* in *Sepia*) fixed in sublimate-alcohol would no longer give a positive Feulgen reaction. But I found that by treating it with OsO_4 before hydrolysis, its staining properties could be restored. I therefore tried the same procedure with sublimate-fixed films of *E. nana*, but the results were again negative.

From all my attempts, therefore, I conclude that the nucleus of *E. nana* contains, in the 'resting' stage, no thymonucleic acid ('chromatin' or 'euchromatin' of modern workers), though its karyosome is composed of 'chromatin' ('basichromatin') in the classical cytological sense.

Finally, the staining reactions of the other parts of the nucleus—apart from the karyosome—must be noted. The clear zone is coloured slightly by 'plasma' stains (eosin, light-green, orange G, etc.), but usually appears almost colourless and structureless. It is tinted pale blue with Mann's stain and Romanovsky methods—the cytoplasm staining a darker blue. The nuclear membrane stains readily with 'chromatin' stains (haematoxylin, carmine, etc., but not Feulgen's reagent), though less intensely than the karyosome. It is coloured bluish purple or lilac with Mann's or Giemsa's stain, while the granules imbedded in it are of the same tint but deeper, or sometimes definitely blue. (The colours vary according to the degree of extraction of the stain during differentiation.)

It will thus be seen that, on tinctorial grounds, there is no reason to suppose that the clear zone contains any 'chromatin'—however this term be defined; while the constitution of the nuclear membrane, with its granules, can be interpreted in various ways. It is unprofitable to discuss these matters further, however, before the behaviour of all the structures during nuclear division has been described.

Notes on Sphaerita.—In my monograph on the amoebae of man (1919, p. 106) I called attention to a curious parasite occasionally present in *E. nana*; and since then many other workers have observed the same (or a similar) organism in many other intestinal amoebae and other protozoa. I have observed this parasite (*Sphaerita*) repeatedly in the course of the present work, and some of my findings seem sufficiently interesting to record.*

Sphaerita† is probably a fungus (Chytridiales), and lives in the cytoplasm—never the nucleus‡—of trophic forms of *E. nana*. It is never found in precystic amoebae or cysts. I have met with it in amoebae of this species living not only in Man but also in several macaques (*M. sinicus* and *M. rhesus*). In all simian infections *Sphaerita* has occurred sporadically and unexpectedly. The case most thoroughly studied is that of my tame monkey Susanna, and may be briefly noted as an example:

Susanna (*M. sinicus*) was naturally infected with *E. nana*, and her infection was studied carefully for 13 years (1924–37), during which time her faeces were examined microscopically and culturally on hundreds of occasions.§ For the first 6 years no specimens of *E. nana* infected with *Sphaerita* were ever seen in them; but infected amoebae then appeared unexpectedly (28. iv. 30), and the infection persisted for 5 days (daily examinations). Thereafter it disappeared, and was never seen again.

The strain of *E. nana* with which I experimentally infected myself (in 1925) was derived from Susanna.‖

* The literature on *Sphaerita* is now extensive, and has recently been fully summarized by Kirby (1941, p. 1040 *seq.*). I shall not discuss it here.

† The genus was proposed by Dangeard (1886) for parasites found in free-living rhizopods (*Nuclearia* and *Heterophrys*). He also found them in *Euglena* and other flagellates. At present it is impossible to assign a specific name to the parasite of *E. nana*, as it is not known whether it is or is not identical with others described and named from other hosts.

‡ A related form (*Nucleophaga*), which parasitizes the nucleus only, has been described from *E. nana* by Nöller (1922), Epstein (1922), and Brumpt & Lavier (1935). I have never encountered it.

§ See preceding instalments of this series.

‖ See Dobell (1933).

My infection has persisted now for over 17 years, and during this time I have made hundreds of examinations of the amoebae in my stools. No *E. nana* infected with *Sphaerita* were encountered for nearly 11 years; and then (28. iii. 34), to my surprise, I discovered some infected specimens. No more were found for 3 months, when a similar observation was made (7. vi. 34). The infection then disappeared once more, and was not seen again till exactly 4 years later (6. vi. 38). Since then no amoebae parasitized by *Sphaerita* have been discoverable.

I have never been able to obtain a cultivable strain of *E. nana* parasitized by *Sphaerita*. I have attempted to do so repeatedly, but the parasites have invariably died out in the primary culture. All amoebae originally infected have invariably produced *Sphaerita*-free strains on cultivation. This is in striking contrast with similar experiments with *E. histolytica*: for I have several times obtained strains of this species in which the infection with *Sphaerita* persisted—in one case for a month (23 serial subcultures, after which the strain was abandoned).*

E. histolytica, as is now well known, is sometimes parasitized by *Sphaerita* when living in Man. But I have also found parasitized specimens of this amoeba in the faeces of macaques, and even in an experimentally infected kitten. (The strain with which this kitten was infected was one long cultivated *in vitro*, and known to be *Sphaerita*-free.) Moreover, the species of *Sphaerita* which parasitizes *E. nana* is probably identical with that which attacks *E. histolytica*: for they are morphologically indistinguishable, and the identification is supported by the following observations:

At the time when Susanna's *E. nana* became infected with *Sphaerita* (*vide supra*, p. 144), she was also infected with *E. histolytica* (a human strain, experimentally implanted, and known to be uninfected with the fungus): but this amoeba became infected at the same time, and infected specimens were found in Susanna's faeces for 13 consecutive days. The parasites then disappeared—as they did from the *E. nana*—and were never seen again. It is thus difficult to escape the inference that both *E. histolytica* and *E. nana* were, in this case, infected by the same parasite.† On another occasion I made a similar observation on the amoebae in my monkey Mungo (also *M. sinicus*).

At the times of the experiments (just noted) on Susanna and Mungo, both were infected with flagellates as well as amoebae. Susanna harboured *Trichomonas* (experimentally introduced), and both monkeys were naturally infected with *Enteromonas*. Neither of these flagellates showed any infection with *Sphaerita* when the accompanying amoebae did, though both flagellates are sometimes found naturally infected. Susanna, for example, at a later date (Nov. 1935) passed specimens of *Enteromonas* infected with *Sphaerita* in her faeces for a brief period; but at this time the amoebae which she then harboured (*E. nana*, *E. histolytica*, *E. coli*) remained exempt. The species of *Sphaerita* which parasitizes *Enteromonas* is therefore probably distinct from that which is found in the amoebae.*

It thus seems probable that the species of *Sphaerita* which parasitizes *E. nana* is the same as that which parasitizes *E. histolytica* (and *E. coli*), but different from that which occasionally attacks *Trichomonas* and *Enteromonas*: but further observations and experiments are needed to settle this question.

It has been suggested by Lwoff (1925) that it might be possible to eradicate *E. histolytica* from the human intestine by introducing *Sphaerita* as a hyperparasite.† My own observations and experiments afford no support for this idea. They even, I think, contradict it: for *Sphaerita* appears to exercise no generally harmful effects upon any amoebic population which it attacks, and its presence in men, monkeys, and kittens does not usually cause the disappearance of either *E. histolytica* or *E. nana*.

(3) *Division*

E. nana multiplies in cultures in only one way—by equal bipartition with mitotic division of its nucleus. Details are not easy to study, and are best seen in specimens fixed by the coverglass technique previously described.‡

Division in the living organism differs in no way from that observable in many other species. A large trophic amoeba, after creeping about actively, comes to rest, and assumes a more or less globular form. Its pseudopodia disappear, though its outline usually remains irregular. After staying quiescent for some minutes, it gradually becomes elongated; and a constriction then appears in the middle—at right angles to the long axis. The constriction slowly deepens, dividing the two halves of the protoplasmic mass, which pull apart and move in opposite directions. The protoplasmic connexion gradually becomes attenuated, and then suddenly snaps. Simultaneously the two daughter amoebae put out pseudopodia and creep away. I have been unable to see any details of nuclear division during this process in the living animal, but in good stained preparations it is possible—though difficult—to study them with some precision.

Prophases.—The earliest stages of nuclear division are some of the most important, but also some of the hardest to study. I have paid much attention to them—as seen in excellent preparations fixed

* This strain of *E. histolytica* was derived from my monkey Polo (*M. sinicus*) after experimental infection from another macaque (Bonar) of the same species. Neither of these hosts had previously shown *Sphaerita*-infected amoebae in their faeces. The infection-experiments have already been described (Dobell, 1931).

† At the time when I found specimens of *E. nana* infected with *Sphaerita* in my own stools (7. vi. 34), I was also myself harbouring *E. coli* (implanted experimentally). This species was also then first observed to be infected with *Sphaerita*—indicating that the parasites in both amoebae are probably identical.

* It is also conspicuously smaller, its spores having only about half the diameter of those seen in *E. nana*.

† The suggestion had actually been made much earlier by Chatton & Brodsky (1909, p. 14, note), in their incomplete but still unsurpassed account of *Sphaerita* from '*Amoeba limax*'.

‡ See Dobell (1942).

and stained in a variety of ways—but am still not wholly satisfied with all my interpretations. The following account is based primarily on specimens stained with iron-alum, phosphotungstic, and molybdic haematoxylin, but results obtained by some other methods will be noted where necessary.

From a study of numerous specimens made by my special technique, it appears that the early prophase stages occur while the amoeba is still creeping about—before it rounds up, and while its pseudopodia are still in action (cf. Pl. I, figs. 3–9). The first signs of nuclear division are shown in figs. 3 and 4. The nucleus swells, and its karyosome contracts into a central mass which stains deeply at the periphery but appears paler in the middle (fig. 3). Its peripheral part seems to be composed of irregular masses of intensely stainable material. At this stage the nuclear membrane is distinct, and the linin threads connecting its granules with the karyosome are clearly seen. Some of the granules in the membrane appear to become detached, so that they come to lie in the perikaryosomic 'clear zone' (fig. 3).

A little later, the nucleus has the appearance shown in fig. 4 (Pl. I). The deeply staining outer part of the karyosome is seen to consist of numerous small granules, while the clear zone contains more abundant and smaller and paler granules, which seem derived—in part, at least—from those originally imbedded in the nuclear membrane (figs. 4, 5). The whole karyosome then appears to disintegrate into minute granules, which become uniformly dispersed and mixed with the granules in the clear zone (fig. 7). Some of the granules stain more deeply than others, but all appear connected by fine linin threads forming an irregular network. I have not succeeded in counting the granules accurately at this stage, but at least 20—and probably more—are usually present. When the karyosome has completely disappeared, some of the resultant 'granules' often appear slightly rod-shaped, or even in the form of short bent filaments. (Cf. fig. 6, which shows an optical section of a nucleus exhibiting such structure.) There is no evidence to indicate that any of the granules are formed from the material which fills the clear zone in the resting nucleus.

At an early stage in the prophases, while the karyosome is disintegrating, a minute deeply staining body becomes visible near the centre of the nucleus. It is similar in size and staining properties to the other granules, and therefore cannot be distinguished from them at first: but it soon divides by constriction into two, and is then recognizable as a minute diplococcus-like or dumbbell-shaped body lying within the area originally occupied by the karyosome (figs. 7, 8). This body is an intranuclear centriole—as is proved by its later behaviour; and although its origin cannot be determined with certainty, it appears to arise inside the karyosome.

The division of the centriole occurs early in the prophases, but does not always synchronize with the formation of the granules in the nucleus. For example, the granules are more uniformly dispersed in the specimen shown in fig. 7 than they are in that shown in fig. 8, though the centriole is at a later stage of division in the latter than in the former. (The figures were selected to illustrate this point. Other variations are to be seen in my preparations.) The intense staining of the centriole may also be emphasized. With haematoxylin, and other stains, it is usually coloured quite as deeply as any of the other 'granules' in the nucleus.

As the daughter-centrioles—formed by division of the originally single centriole—draw apart, they remain connected by a conspicuous centrodesmus. At the same time, the granules become fewer, and are seen to consist of two sorts—a group more deeply staining and centrally situated, and a group of paler units at the periphery (fig. 9). The darker granules—as shown by their subsequent behaviour—are chromosomes. The paler granules ultimately vanish, and appear to play no part in mitosis. Whether the chromosomes (dark central granules in fig. 9) are formed by fusion of some of those present at earlier stages (fig. 7), or whether they are derived from a few of these granules, while the rest are resorbed, I am not able to determine.

The stage shown in fig. 9 is followed immediately by that shown in figs. 10, 11. The daughter-centrioles—connected by the conspicuous centrodesmus—pass to opposite poles of the nucleus; the chromosomes arrange themselves in a ring on the equatorial plate; and the pale peripheral granules disappear. Spindle-fibres are now first seen joining the centrioles to the plate (though their attachments to the chromosomes are indistinct), and in this way an intranuclear mitotic spindle is formed. This is the stage at which the amoeba becomes rounded and at rest; and it remains in this condition for a relatively long time, apparently, as the spindle-figure (equatorial plate stage, figs. 10, 11) is usually the commonest division-stage found in preparations. It is highly characteristic of *E. nana* and we may therefore pause with it to consider it in more detail.

As will already be evident, the spindle formed between the centrioles is entirely intranuclear. The centrioles are in contact with the nuclear membrane, and there are no astral radiations extending into the cytoplasm. The membrane stains distinctly at this stage, but appears somewhat attenuated and may appear slightly granular in places. The chromosomes are always arranged in a ring round the equator. They are very small, slightly unequal in

size, and usually not quite regularly disposed. As a rule some of them are in contact, and as the ring is generally seen edgewise they are extremely difficult to count (cf. fig. 10). Whenever they are well separated, however, and the counts are certain (cf. fig. 11), I have almost invariably been able to make out 10; and this I believe to be their true number.*

The chromosomes stain intensely with iron-haematoxylin (and all other haematoxylin stains), with carmine, safranin, Mann's stain, and Romanovsky methods. With Mann's or Giemsa's stain they are coloured an intense purple. Moreover—and this is remarkable— the centrioles are usually coloured as intensely as the chromosomes, and of the same shade, by both methods. The spindle-fibres are usually tinted lilac or pink—not blue—by Mann's stain and also by all Romanovsky methods tried (Giemsa, Leishman, etc.). The spindle itself is not a solid body—as in some other amoebae—but a figure formed by comparatively few fibres radiating towards the equatorial plate from the polar centrioles.

It was to be expected that the Feulgen reaction would assist in the elucidation of the prophases, by colouring the 'chromatin' granules and chromosomes electively—and thus distinguishing them from all the other 'granules' present in the nucleus at early stages. But none of the granules give a positive 'nucleal reaction' (in my hands). All nuclei at the stages shown in figs. 3–11 appear completely negative. The only exception observed is the equatorial plate stage (figs. 10, 11), at which sublimate-fixed material treated afterwards with 'osmic acid'† occasionally shows a very faint coloration of the chromosomes. I cannot call it a positive reaction.‡ Variation in the times of hydrolysis and staining§ has failed to intensify it.

Mann's stain and various Romanovsky methods have also failed, in my hands, to differentiate the 'chromatin' from other prophase granulations. With both methods all granules stain the same colour—the only difference being that some may be stained rather more deeply than others.

Metaphase.—The chromosomes round the equatorial plate divide by constriction into two daughter-plates. Details are extremely difficult to make out, and even more difficult to delineate. Fig. 12 is an attempt to depict a metaphase nucleus in which the chromosomes can be counted with some certainty in the actual specimen. There are here two daughter-rings (10 chromosomes in each) close together, and some of the daughter-chromosomes can be seen still connected—lying like tiny dumb-bells in the long axis of the spindle. At this stage the nucleus is somewhat elongated, and the polar centrioles, with their connecting centrodesmus, are very distinct.

Anaphases.—As the daughter-groups of chromosomes separate and pass towards the poles of the spindle, they lose their annular arrangement and become irregularly scattered (figs. 13, 14). In the early anaphases they can often be counted with considerable certainty, and their number is again seen to be 10. This stage is of brief duration, however, and they soon collect at the poles in an unresolvable mass. The nucleus itself now becomes fusiform, with the more widely separated centrioles projecting into the cytoplasm (fig. 15). In later anaphase the nucleus becomes still more elongated, and the chromosomes appear larger and more aggregated (fig. 16). Throughout these stages the centrodesmus is usually very conspicuous (figs. 15, 16), but the nuclear membrane gradually disappears. No spindle-fibres can usually be detected, and the centrioles become indistinguishable among the chromosome clumps.

Telophases.—The anaphase chromosomes separate still further, and appear to fuse into a few irregular but intensely staining masses (fig. 17). The nuclear membrane is no longer visible, but a clear zone now appears round each chromatin mass; and as the organism elongates and begins to constrict into two, new membranes form round the daughter-nuclei (figs. 17, 18). The centrodesmus remains distinct though much attenuated during all these stages, but its terminal centrioles are usually invisible.

When the cytoplasm undergoes constriction, the daughter-nuclei have the form shown in fig. 19. The chromosomes are now transformed into a few densely staining blocks, closely aggregated to form the new karyosomes. The clear zone surrounding them is well developed, and the new nuclear membranes thin but definite. At the moment of separation of the two daughter-amoebae (fig. 20) their nuclei are completely reconstituted. The karyosomes—formed by fusion of a few large chromatin masses, derived from the chromosomes—lie centrally, surrounded by a clear zone bounded anew by a nuclear membrane. Traces of the centrodesmus may still be seen, but the centrioles have vanished. After separation (fig. 21), the karyosomes appear massive and central, and the linin threads uniting

* For a long time I believed there were only 8; but as I obtained better and better preparations I was able to distinguish at least 9 or 10 in many specimens. All the best specimens, when accurately studied and drawn with the *camera lucida*, reveal at least 10. There are certainly more than 8 and less than 12, anyway.

† Cf. p. 144 *supra*.

‡ It is so faint that I can only convince myself of it by examining the preparations in monochromatic green light. With such illumination they appear slightly more distinct than with ordinary white light.

§ Cf. p. 143 *supra*.

them to the membrane again become evident.* Soon after they creep apart the daughter-amoebae begin to show all forms of karyosome characteristic of trophic amoebae.

Throughout the anaphases and telophases (figs. 14–21) the chromosomes, chromatinic masses, and reconstituted karyosomes stain intensely (red or purple) with Mann's and Giemsa's stains—exactly like the karyosomes of 'resting' nuclei. But at all these stages they remain negative to the Feulgen stain, though they colour readily with the counter-stains (light-green or orange G) employed in this technique. The Feulgen reaction is thus negative at all stages of nuclear division with the possible exception of the chromosomes on the equatorial plate.

The interpretation of these findings will be considered again later.†

(4) *Precystic amoebae and encystation*

Precystic amoebae—that is, those which give rise to cysts—are smaller than typical trophic amoebae, and present other peculiarities. They are formed in the following way:

At the penultimate division before encystation, the daughter-amoebae do not grow but remain small—though their nuclei are of normal size and structure. These amoebae then divide into two, so that they produce still smaller amoebae with relatively large nuclei. The nuclear division appears to differ in no way from that of ordinary trophic forms, and therefore needs no further description. Prophase, equatorial plate (spindle), and telophase stages are illustrated in figs. 22–25. The small precystic amoebae formed by this division (fig. 25: cf. fig. 20) are very characteristic. They differ not only in absolute size but also in the relative size of their nuclei, and in their cytoplasmic structure. They contain no food-vacuoles, as all ingested bacteria are assimilated and no others are eaten at this stage.

Precystic amoebae are often extremely small, sometimes measuring no more than 3·5 μ in diameter (cf. fig. 29).‡ But they creep about actively for a time, and show most of the polymorphic forms of karyosome seen in trophic amoebae. (Typical examples are depicted in figs. 26–28.) In haematoxylin preparations they often—though not invariably—display a vacuole (figs. 26, 28, 30), which contains glycogen during life. It stains brown with iodine (in fresh specimens), red with Best's carmine, and pink with chlorazol black. Many precystic amoebae also show already (fig. 30) a few small cytoplasmic granules, which are the 'volutin' granules subsequently seen in the cysts. At this stage, however, it is not always possible to distinguish these from the remains of previously ingested bacteria. In the living organism they are bright and refractile, and stain with neutral red. In fixed preparations they stain with haematoxylin, though less intensely than the nuclear chromatin, and usually appear to lie in minute vacuoles. With methylene blue they stain metachromatically (red).

A typical precystic amoeba (figs. 26, 27, 28, 30) therefore differs from an adult trophic amoeba by its smaller size, relatively large nucleus, clear cytoplasm devoid of bacterial inclusions, and sometimes by the presence of a glycogen vacuole and volutin granules: but its nucleus may show almost any of the karyosomic forms seen in trophic amoebae. It turns into a cyst by rounding itself into an oval or spherical mass, withdrawing its pseudopodia, and secreting a delicate cyst wall (figs. 31, 32). At the time of encystation the karyosome usually appears massive and globular, but its position may be either central or eccentric in the nucleus (cf. figs.).

(5) *Cysts*

The cysts of *E. nana* are already well known, and have often been described.* They are typically oval or spherical, and measure about 6–9 μ in length and 5–7 μ in breadth. Larger and smaller specimens also occur—the smallest being only about 4 μ in diameter, and usually spherical. The large 'oval' cysts are seldom symmetrical—one side being more convex than the other, so that they appear gibbous. The cyst wall is uniformly thin, smooth, and colourless. Cysts formed in cultures differ in no way from those passed in the faeces of men and macaques.

The cyst, when first formed, contains a single nucleus (with large globular karyosome) and usually a glycogen vacuole (sometimes two) and a variable number of minute 'volutin' granules (fig. 32). There are no chromatoid bodies or other inclusions. The nucleus soon divides into two, and each daughter-nucleus, after a short period of rest, again divides. Both divisions are mitotic, and appear to be closely similar to the divisions seen in trophic amoebae: but as the nuclei become smaller at each division, the details become increasingly difficult to study. The centrioles are conspicuous, but I have been unable to count the chromosomes accurately at either division. There is no evidence that they

* The specimens shown in fig. 21 appear somewhat too large, in comparison with the other figures. This is because they were much flattened out on the coverglass; but I have drawn them on purpose, as they show every detail of nuclear and cytoplasmic structure at this stage (nothing visible has been omitted from the drawing) with diagrammatic clarity.

† See p. 154 *infra*.

‡ I have previously stated that precystic amoebae 'are not distinctly smaller than ordinary active forms' (Dobell, 1919, p. 106). This is an error—based on a study of faecal specimens only.

* Cf. Dobell (1919), p. 106.

undergo reduction. The glycogen vacuole persists at all stages, and usually attains its maximum size when the cyst is binucleate. I have illustrated these stages in figs. 33–40, drawn from typical specimens, and can add little more to these pictorial descriptions (which the interested reader is invited to study carefully).

The ripe cyst (fig. 41) usually contains 4 small resting nuclei—each with a massive and generally eccentric karyosome, and a distinct membrane. In good-sized specimens the nuclei are about 1·5 μ in diameter. Most mature cysts contain no glycogen, but a variable number of volutin granules. Their cytoplasm is otherwise clear.

The size of the cyst is proportionate to that of the precystic amoeba from which it is formed. Very small amoebae (fig. 29) produce very small cysts (figs. 44, 45), large individuals large ones. But all—if normal—appear to be equally viable. Supernucleate cysts may, however, be formed in cultures as they are in the intestine, and their occurrence must therefore be noted here.

Supernucleate cysts—containing more than the typical 4 nuclei—are formed by the division of one or more of the nuclei normally present in the ripe cyst. When only one nucleus divides it gives rise to a cyst containing 5 nuclei (fig. 42): when all four divide a cyst with 8 nuclei results (fig. 43). Intermediate forms also occur, but all are rare in cultures—as they are in faeces. Cysts containing 8 nuclei are always abnormally large.*

Glycogen—as already noted—is normally present in the cysts of *E. nana* during their development but not in mature (4-nucleate) specimens.† It can be readily demonstrated not only with iodine in fresh cysts but also in fixed cysts by staining them with chlorazol black. In these (after fixation in Schaudinn's fluid) the glycogen vacuoles appear to be filled with clouds of minute pink granules; and their appearance is thus different from that seen in iodine-preparations, where the glycogen stains more homogeneously. It may be noted further that the glycogen is normally more plentiful in culture-cysts than in those passed in the faeces (as it is in *E. histolytica* and *E. coli*), and that it disappears rapidly from all cysts kept at low temperatures. Both glycogen and 'volutin' are reserve materials—the former being normally used up during development, and not stored during extracorporeal or extracultural life.

Karyosomic polymorphism—so characteristic of the 'resting' nuclei of trophic amoebae—is not usually seen in the nuclei present in cysts. At all 'resting' stages in these (1-, 2-, 4-nucleate) the karyosomes are globular, massive, and often eccentric, though they sometimes show one or two smaller accessory masses or granules attached by fine threads. (Cf. figs. 32, 36, 40, 41.) In the ripe cyst, the karyosomes of the nuclei are the most conspicuous objects. Some of the larger 'volutin' grains, however, may be equally deeply stained, and readily catch the eye. On superficial examination, therefore, they may be mistaken for karyosomes of supernumerary nuclei—an error detectable in some previous descriptions.*

Finally, it may be added that cannibalism—which is not uncommon in cultures of all the intestinal amoebae of men and macaques—appears to be particularly prevalent at precystic stages of *E. nana*. The small precystic amoebae are not seldom ingested by the larger trophic forms simultaneously present (fig. 48). Trophic amoebae even ingest cysts occasionally (fig. 49): and in both cases they are able to digest their prey. I have specimens showing all stages in the process, but have not thought it necessary to depict them.

Note on filamentar inclusions.—In my monograph of 1919 I drew attention to certain peculiar inclusions which are sometimes seen in the cysts of *E. nana*. I noted (p. 108) that these are sometimes long and filamentar, and sometimes in the form of bundles of short rods or heaps of coccus-like granules. The inclusions are visible in living cysts, and stain readily with iron-haematoxylin; and they appear to be characteristic of the infections in certain human beings. At that date (1919) I was unable to interpret these inclusions with certainty, but suggested that they might be symbiotic or parasitic bacteria, or possibly structures like the chromatoids of *E. histolytica*.

In the course of further work I have encountered these inclusions repeatedly, and have been much perplexed by them. They occur not only in the cysts of human strains of *E. nana*, but also in those derived from macaques; and they may be seen from time to time both in cysts discharged naturally with the faeces and also in those formed in cultures.

Although these inclusions are still problematic, I now feel fairly confident that they are parasitic micro-organisms. Evidence for this interpretation is supplied by the following facts: (1) The inclusions are not constantly present in every strain. From some, indeed, they appear to be entirely absent; and in those strains in which they occur, the proportion of 'infected' cysts (both in cultures and in faeces) may vary considerably. (2) The uniform size and form of the inclusions in many cysts strongly suggests that they are bacteria. In newly formed cysts, for example, they often have the regular shape and size of bacilli (cf. fig. 46, Pl. I). (3) They appear to become larger and more abundant in ripe cysts kept at low temperatures, and after incubation at 37° C. they often appear to grow into long filaments (cf. fig. 47, Pl. I). This is incompatible with the view that they are chromatoid bodies. (4) Cysts containing these inclusions usually appear more or less degenerate.

* Cf. Dobell (1919), p. 108, and fig. 86, Pl. V, *ibid.*

† Noted earlier (Dobell & Jepps, 1917: Dobell, 1919, p. 107), but overlooked by most other observers.

* Cf. Dobell (1919), p. 108.

Their cytoplasm is frequently vacuolated, and their nuclei abnormal (fig. 47). (5) So far as I have been able to ascertain, cysts containing these inclusions never hatch. They invariably degenerate and die on incubation.

From all these considerations I conclude that the 'filamentar inclusions' are not normal elements in the cysts of *E. nana*, but parasitic bacteria which usually cause their death. But I have hitherto been unable to cultivate them separately, or to identify them with any of the micro-organisms present in the intestinal contents or in cultures of this amoeba.

(6) *Excystation*

The process of excystation is difficult to observe in *E. nana*, owing to the small size of the cysts and the capricious way in which they often behave in cultures. Moreover, the finer details can be properly studied in first-rate stained preparations only, and these are far from easy to obtain.

When ripe normal cysts of *E. nana* are incubated at 37°–38° C., in suitable medium and in the presence of appropriate bacteria, they hatch after the lapse of several hours and each liberates a single small amoeba. No division of nuclei or cytoplasm occurs in the cysts, and the times and manner of their hatching are closely similar to those already described for *E. histolytica*.* If the cysts are inoculated into medium at room-temperature, and then placed in the incubator immediately, some of them will usually hatch in 4–5 hours. After 5–7 hours most of those which can hatch have usually done so, but excystation in individual cases may be delayed for much longer periods (up to about 18 hours).

During the first few hours of incubation the cyst undergoes certain changes. At first it contains (fig. 50, Pl. II) no visible structures except its four characteristic nuclei and a few 'volutin' grains, imbedded in a homogeneous cytoplasm which entirely fills the cyst. No glycogen is normally present. The 'volutin' grains are refractile and easily visible in the living cyst, but the nuclei are inconspicuous. In stained cysts, however, they are the most noticeable objects, and appear as small vesicles with relatively large but often eccentric and somewhat irregular karyosomes. The nuclear membranes are delicate but distinct, and the 'clear zone' around the karyosome appears pale and structureless. But the 'volutin' granules gradually disappear on incubation, and the cytoplasm then becomes pellucid and homogeneous. It also stains more palely, and the nuclei thus stand out more prominently, while their karyosomes now appear more rounded and central (fig. 51).

A little later, the cytoplasm begins to contract from the cyst wall, and at the same time becomes denser. The nuclei also usually seem to become slightly smaller with the shrinkage of the cytoplasm (fig. 52). The space between the cytoplasm and the cyst wall gradually extends, and the cyst contents contract still further. Protoplasmic streaming is visible at this stage in the living organism, and the nuclei can be seen to share in the movement. A little later still, the whole protoplasmic mass is seen to be detached from the cyst wall, and to lie freely in the cyst as a small amoeba (fig. 53). This soon shows distinctly differentiated endoplasm and ectoplasm, and begins to move about inside the cyst by means of a few large pseudopodia. The endoplasm now stains more deeply, and cysts at this stage have therefore a very characteristic appearance (cf. figs. 51, 53).

The amoeba moves about inside the cyst for some time—its clear ectoplasmic pseudopodia continually stroking the inner surface of the cyst wall. This, though very thin, hardly changes its shape with the movements of the amoeba: it appears rigid but slightly elastic, and consists of only one uniform layer with no pores or other visible structures. After a time—just as in *E. histolytica*—the amoeba comes almost to rest, with one of its pseudopodia pressed against a point on the inner surface of the cyst. Shortly afterwards a minute bead of protoplasm appears at the tip of the pseudopodium *outside* the cyst, and enlarges into a small globule. Even in stained preparations it is impossible to see how the cyst wall is perforated. The pore* through which the protoplasm passes is so minute that it is not distinctly resolvable. All that can be seen at these stages is the external bead of cytoplasm connected by a delicate stalk with the amoeba inside the cyst (see figs. 54, 55).

When the external globule of protoplasm has increased somewhat in size, it becomes smaller again. The partly emerged protoplasm enters the cyst once more, leaving only a minute bead outside: and this process is then repeated—more and more of the organism emerging as a hernia-like mass at each attempt. A half-emerged amoeba has the appearance shown in fig. 56; and this stage, in life, would probably be followed again by that shown in fig. 55. Sooner or later, however, the whole amoeba squeezes itself through the pore—even the nuclei being somehow able to pass through its very tight constriction. They escape one at a time, with successive outflowings of the protoplasm, until the whole organism lies outside the cyst (figs. 57–59). The newly-hatched amoeba then usually rests on the outside of its cyst for a few minutes (fig. 59) before it puts out pseudopodia and creeps away actively (figs. 60, 61).

* See Dobell (1928), p. 383 *et seq.*

* It appears to have no definite location; for I have seen specimens in which it is situated at almost any point in the circumference of the cyst wall.

When the amoeba has left its cyst, the latter retains its shape and appears perfectly empty save for a few minute granules—highly refractile in life, but usually unstained in permanent preparations—which are left behind (cf. figs. 60, 61). Occasionally, however, a small bit of protoplasm—nipped off from the 'tail' of the amoeba as it makes its final escape—may remain in the cyst. (It is sometimes as large as the intracystic mass of cytoplasm shown in fig. 58.) After excystation, no trace of the pore, and no breach of any sort, can be seen in the cyst wall, which appears to remain quite intact. (Cf. figs. 58–61.) Empty cysts are easily recognizable in cultures at the time of hatching, but they dissolve and disappear soon afterwards.

During all stages of excystation the nuclei are irregularly distributed through the cytoplasm: but when the amoeba has fully emerged they run together into a compact clump—just as they do in *E. histolytica*—so that the hatchling, with its 4 agglomerated nuclei, is a very characteristic form (figs. 60, 61, 63–66). As soon as it hatches, moreover, the amoeba begins to feed, and consequently its endoplasm is soon filled with small ingested bacteria (cf. figs.).

The nuclei frequently display irregularities in size and shape during excystation, doubtless owing to the strains and stresses to which they are subjected: but in the newly hatched amoeba they recover their form and appear as 4 uniform and equal-sized vesicles, with relatively large, globular, and central karyosomes, and with delicate but distinct membranes (fig. 65, etc.).*

Even very small cysts (measuring no more than 5μ in diameter) appear to hatch normally, but it is impossible to make out many details in them. They produce proportionately small amoebae—of typical structure, with 4 tiny nuclei—which may be only 3–4μ in diameter when rounded. A small specimen of this sort is shown in fig. 62 (cf. figs. 60, 61).

Supernucleate cysts (cf. p. 149) are viable, and able to hatch: but I have been able to observe the chief stages in 5-nucleate cysts only. Fig. 68 shows a 5-nucleate amoeba loose inside its cyst (cf. fig. 53), and fig. 69 shows a similar form which has just emerged and is sitting on its empty capsule (cf. fig. 59). The smaller size of 2 of the nuclei (formed by division of 1 of the original 4) is noticeable in these specimens. When the 5-nucleate amoeba has fed and grown, however, all its nuclei appear of equal size. Fig. 70 illustrates one of the few specimens which I have hitherto succeeded in finding. Its nuclei are in contact but not agglomerated (as in 4-nucleates): but I am unable to say that this is a typical arrangement, as I have seen so few.*

Other supernucleate cysts doubtless also hatch, and liberate amoebae with nuclei corresponding in number to those originally present in the cyst. But I have been unable to study all stages, and base this conclusion on the observation of supernucleate metacystic amoebae (see p. 153 *infra*). At all events, I have no evidence that infranucleate cysts—like those so commonly seen in *E. coli*†—are ever formed by *E. nana*.

(7) *Metacystic development*

The metacystic development of *E. nana* is, in reality, extremely simple, but has proved extraordinarily difficult to study because of the very small size of the amoebae and the misleading appearances of their nuclei at many stages.

Development takes place, briefly, as follows: Each quadrinucleate amoeba which hatches from a cyst divides—by successive cytoplasmic bipartitions with no nuclear divisions—into 4 uninucleate amoebulae, which grow up into ordinary trophic forms. At each cytoplasmic fission the nuclei are distributed at random to the daughter-individuals, so that several different lines of development may be followed. Moreover, before and after division the metacystic amoebae may undergo growth, so that individuals of identical nuclear constitution may be of very different sizes. They may thus contain 1, 2, 3, or 4 nuclei, and be of any diameter from only 2–3μ (1-nucleate amoebulae) up to that of trophic forms.

It will be clear, from the foregoing outline, that the 4-nucleate metacystic amoeba (hatched from a cyst) may develop in two different ways. It may divide (i) into a trinucleate and a uninucleate, or (ii) into two binucleate amoebae. In the first case (i), the 4-nucleate (e.g. fig. 65) begins its division by the separation of 1 of its clumped nuclei from the other 3 (which remain in contact, fig. 71). The organism then elongates and constricts into two roughly equal parts (fig. 72)—one containing 3 nuclei, the other 1. A trinucleate amoeba, with 3 adherent nuclei (fig. 73), and a uninucleate amoebula (fig. 74) result. In the second case (ii), the 4 clumped nuclei of the quadrinucleate (e.g. fig. 65) separate into 2 pairs: the organism then elongates and is constricted into 2 binucleates (figs. 85, 86).

Every trinucleate amoeba (figs. 73, 75) divides into a binucleate and a uninucleate amoebula by

* The 4 nuclei do not usually lie in one plane, but are closely packed to form a tetrad. When seen under the microscope, only 3 are usually visible at once—the fourth lying over or under them. They are not easy to depict, therefore, and the specimens illustrated were selected because their nuclei happened to lie conveniently separated.

* The specimen figured may possibly be the metacystic product of a supernucleate cyst which originally contained more than 5 nuclei.

† See Dobell (1938), p. 206.

a like process (figs. 76, 77). The products are shown in figs. 78 and 79.

A binucleate amoeba (fig. 87), however formed (cf. figs. 77, 86), always divides by separation of its nuclei, elongation, and constriction of its cytoplasm (figs. 88–90), into 2 uninucleate amoebulae.

During all these divisions the nuclei remain at rest. They have the same form—with spherical and central karyosome—as is seen in the 4-nucleate amoeba which escapes from the cyst (cf. figs.).

Some of the amoebulae formed during early metacystic development are often excessively minute. They may measure no more than 2μ in diameter when rounded (fig. 91), and are thus even smaller than the nuclei of ordinary trophic organisms. Yet they are perfect little animals, with normal nuclei and pseudopodia, and grow into adults like their bigger brethren (figs. 74, 79).

As will be evident from the figures, the nuclei in trinucleate and binucleate amoebae—as in quadrinucleates—are always closely aggregated. They separate only at the time of division, when they appear to lose their attraction to one another.

Interpretation of the metacystic stages would be easy if the forms just described and illustrated were the only ones found in early cultures made from cysts. But unfortunately they are not. Between divisions, the amoebae feed and grow—to a variable extent—and their nuclei undergo many changes of form. Their karyosomes become polymorphic, and may show many of the modifications seen in trophic amoebae.* Consequently, the nuclei of metacystic amoebae often present the most puzzling appearances, which may easily lead to misinterpretations. These must now be briefly considered.

Quadrinucleate amoebae, when not dividing, may have all their nuclei like those shown in figs. 63–65—with compact central karyosomes: or they may show the karyosomes peripherally distributed, so that all nuclei appear annular in optical section (fig. 67). All intermediate forms also occur. Sometimes the quadrinucleates grow into large amoebae—as big as trophic organisms—before they divide: and they then show a variety of remarkable appearances which can easily be misunderstood. A form such as that depicted in fig. 101, for example, with the chromatin in its nuclei arranged in a small central mass and a larger layer lining the nuclear membrane, might easily be mistaken for a recently hatched small specimen of *E. histolytica*.† Trinucleate amoebae, with their nuclei in a similar condition (fig. 100), might likewise be mistaken for metacystic forms of *E. histolytica* or even of *E. coli*.‡

But the most misleading appearances are presented by binucleate amoebae. Those with round, central, and homogeneous karyosomes (figs. 78, 80, 87), offer no difficulty; but those with irregular (fig. 82) or peripherally distributed (fig. 83) karyosomes are at first sight less easy to interpret. When the two nuclei—always closely appressed—have such karyosomic arrangements as are illustrated in figs. 81 and 84, they may very readily be mistaken for stages of division in a uninucleate amoeba. The specimen depicted in fig. 84, indeed, looks under a lower power exactly like an amoeba with a single nucleus at the metaphase of mitosis. The 'centrioles' and split 'equatorial plate' are obvious: but the specimen is really a 'resting' binucleate amoeba with both its closely applied nuclei in the karyosomic condition depicted in fig. 2 (Pl. I) or fig. 98 (Pl. II).

I have not figured all the various nuclear conditions which can be seen in metacystic amoebae, but only a selection. In figs. 93–97, however, I have illustrated various nuclear appearances commonly observable in amoebulae which are in process of growth into trophic amoebae. Larger specimens—with more compact karyosomes—are shown in figs. 92, 98, 99. Careful examination of all these figures will, I hope, obviate the necessity for further description in words.

It is noteworthy that, as a general rule, the nuclei of multinucleate amoebae always have similar karyosomic arrangements. When the karyosome is central and massive in one nucleus, it is so in all (figs. 65, 75, 80): when it is peripherally distributed, all the nuclei have a similar structure (figs. 67, 101, 100, 83): and so on for other forms of karyosome. Quadrinucleates with central karyosomes in some nuclei and 'annular' chromatin in others are never seen, nor binucleates with two unlike nuclei.

In cultures made from cysts, all the different forms of metacystic amoebae depicted on Plate II may be found at almost any time during the first day of incubation. Excystation usually begins in about 5 hours, and the first metacystic divisions may occur shortly afterwards. In cultures only 6 hours old, therefore, amoebae with 1, 2, 3, and 4 nuclei may commonly be found. At first 4-nucleates predominate: later, 3-nucleates and 2-nucleates, and finally 1-nucleate amoebulae, become increasingly frequent. As all these forms creep about actively, feed and grow and divide, and display a great variety of nuclear conditions, the array of organisms encountered in young cultures is at first sight extremely puzzling. It would be impossible, indeed, to understand them all correctly without seeing the various division-stages, and without knowledge of the remarkable transformations which the 'resting' karyosome undergoes in trophic amoebae. These supply the clues to interpretation. But dividing organisms are rare in most permanent preparations, as the process is evidently rapid; and

* Cf. p. 141 *supra*, and Text-fig. A (p. 142).

† Cf. Dobell (1928), Pl. XXIII, figs. 36, 37, etc.

‡ Cf. Dobell (1938), Pl. VIII, figs. 37, 39.

living amoebae are even more difficult to study owing to their annoying habit of congregating in clumps and taking cover in dense masses of bacteria. *E. nana* is, indeed, the most troublesome of all the intestinal amoebae of Man to study accurately in its metacystic stages.

I may now summarize the chief points noted in preceding paragraphs in the following synopsis of the metacystic development as a whole. (It must be remembered that the amoebae may be of various sizes, at all stages, owing to growth during inter-division-periods; and that their nuclei at these times may show almost any of the karyosomic distributions seen in trophic amoebae. Their karyosomes are always spherical and central at the moment of cytoplasmic division, but not necessarily so at other times: and no *nuclear* divisions occur at any stage.)

METACYSTIC AMOEBAE are of 4 sorts:*

A. **Quadrinucleates** ($4N$), which hatch from normal 4-nucleate cysts. Each gives rise by binary fission to a trinucleate ($3N$) and a uninucleate ($1N$) or to two binucleates ($2N$).

B. **Trinucleates** ($3N$), formed by fission from A. Their progeny are binucleates ($2N$) and uninucleates ($1N$).

C. **Binucleates** ($2N$), formed by fission from A or B. By division each produces 2 uninucleates.

D. **Uninucleates** ($1N$) are formed by fission from A, B, or C. They are the *amoebulae* which grow ultimately into ordinary trophic amoebae.

It should be emphasized here that all the final products of the quadrinucleate amoeba which escapes from a cyst are alike: they are little uninucleate amoebae (amoebulae) which grow into big trophic organisms directly. They are not gametes, and no conjugation or fusion of individuals or nuclei occurs at any stage (though some binucleate forms, such as figs. 80–82, might suggest at first sight a nuclear fusion). And as there is no evidence of chromosomic reduction during cystic divisions, while no nuclear divisions at all occur during the metacystic development, it may be concluded that there are no sexual stages in the life-history of *E. nana*. The whole life-cycle of this species—like that of *E. histolytica* and *E. coli*—thus appears to be completely asexual.

It remains to say a further word about the development of supernucleate cysts. Unfortunately, I have been unable to determine this in every detail.* As already noted (p. 151), 5-nucleate cysts are able to hatch, and produce 5-nucleate amoebae. I have not succeeded in finding the division-stages of these forms; but as they could divide only into amoebae containing 1, 2, 3, or 4 nuclei, their products would be indistinguishable from those arising from normal 4-nucleate cysts. The only dividing metacystic amoeba with more than 4 nuclei which I have yet found in my preparations is that depicted in fig. 102 (Pl. II). This is a 6-nucleate dividing into 2 trinucleates. It may be the product of a 6-nucleate cyst, or of an 8-nucleate which has already undergone one division (into a $6N$ and $2N$). I figure it as an interesting curiosity.

IV. DISCUSSION

Since I analysed all the then known facts about the cytology and life-history of *Endolimax nana* 24 years ago†, next to nothing has been added to our knowledge of these subjects. For nearly a quarter of a century they have been generally neglected.‡ Consequently, there is not much to discuss now except my own further work—here first reported—which will doubtless receive due criticism and correction from others in the course of time. I may be permitted, however, to comment upon it myself at this point, and to bring it into line with previous researches. The time is not ripe for full discussion, and my remarks must therefore appear somewhat disjointed and inconsequent.§

First, I will emphasize again the extremely small size of *E. nana*, for this entails errors of observation which are almost unavoidable. The smallest metacystic amoebae of this species are probably, indeed, the smallest amoebae known. I am aware, of course, that Vahlkampf, many years ago,‖ described even

* In this synopsis, supernucleate forms are left out of account. The letter N, denoting an undivided cystic nucleus, has the same significance as in my previous symbolic notation for *Entamoeba histolytica* and *E. coli*.

* On analogy with *E. histolytica* and *E. coli*—in which I have followed the metacystic development of supernucleate cysts in some detail—it seems probable that the comparable forms of *E. nana* are viable, and able to develop in the manner to be predicted: but the rarity of all these forms makes verification of this supposition extremely difficult.

† Dobell (1919), p. 101 *et seq.*

‡ I say this in all ignorance and humility. I have made every effort to ascertain what other workers have done, but under present conditions it is impossible for me to know everything that may have been published since 1939.

§ The benevolent reader will (I hope) forgive me if, in these days of paper shortage, I do not again discuss—or even refer to—certain recent publications which merely revive mistakes already corrected.

‖ Vahlkampf (1905), who studied a free-living amoeba which he identified (incorrectly) as '*Amoeba limax*', says his organisms measured only 0·75 μ by 1·5–4·0 μ, with nuclei 0·3–0·5 μ in diameter. (Its cysts were said to measure only 1·5 μ in diameter.) As Alexeieff (1911) has pointed out, these impossible figures should be multiplied by at least 8 or 10.

smaller organisms; but there can be no doubt that his recorded measurements were erroneous.

Apart from its small size, the most remarkable feature of *E. nana* is its nucleus. I described this carefully in 1919, and have now redescribed it in greater detail. My interpretations have not been accepted by some writers, however, so a further word seems called for here. I need not discuss everything that has been written, but will take two typical examples of the criticisms which have been made.

Nöller (1922)—followed by others—considers that the karyosome of *E. nana* is not polymorphic, as I describe it, but normally a compact spherical mass. The polymorphic and variable structures which I describe in trophic amoebae are, he considers, 'with great probability to be interpreted as degenerative' (p. 151). I can only beg him—and others who still hold similar views—to re-examine this question with more care. I feel confident that this will necessitate reinterpretation in agreement with my conclusions.

Stabler (1932), on the other hand, believes that the nuclear structure which I have described in *E. nana* is an artifact due to faulty fixation. He claims that if amoebae (in faeces) are fixed in 'Schaudinn's fluid plus 20 per cent glacial acetic acid', the karyosome appears 'faint and careful observation reveals a series of chromatin lumps on the periphery of the nucleus': and from this he deduces that the true nuclear structure of *Endolimax* is closely similar to that of an *Entamoeba*. In my hands, however, Stabler's fixative does not produce the appearances described. I find it a poor fixative for amoebae, and am at a loss to understand why his alleged results should be preferred to those constantly obtained by more acceptable methods.

Nevertheless, interpretation of the various elements in the trophic nucleus of *E. nana* is not easy. I have recorded my own observations in some detail, but find it hard to reconcile or even to compare them with those of others on other species of amoebae. And I find it particularly difficult to determine which elements are composed of 'chromatin', because different workers define this substance in different ways—some attaching crucial importance to the results of particular staining methods, such as Mann's stain, or Giemsa's stain, or Feulgen's 'nuclear reaction'. But the evidence afforded by these methods is unfortunately inconsistent. Most of the previous discussion, moreover, has centred round the small free-living amoebae commonly miscalled '*Amoeba limax*' or 'amoebae of *limax* type', which I believe to be but remotely related to *E. nana*.

Thirty years ago these small '*limax* amoebae' were generally described as having a vesicular nucleus with a large central chromatinic karyosome, surrounded by an achromatinic clear zone (sometimes containing peripheral achromatinic granules). The chromosomes were believed to be formed mainly from the chromatin in the karyosome.* This interpretation was afterwards reversed by Wasielewski & Kühn (1914), who brought forward strong evidence to show that the karyosome—though it stains intensely with haematoxylin and other basic dyes—contains no 'chromatin'. This, they believe, is present only in the 'achromatinic' clear zone and peripheral granules; and it is from this part of the nucleus—not the karyosome—that the chromosomes are formed. It is to be noted that this view rests largely on results obtained by Romanovsky staining methods.

The conceptions of Wasielewski & Kühn are now widely accepted,† and it might thus have been anticipated that later workers, armed with the Feulgen technique, would readily confirm them. But this has not happened. True, some careful workers‡ have succeeded in demonstrating 'chromatin' in the peripheral zone, and none in the karyosome: but others, apparently equally careful,§ have failed to obtain a nucleal reaction in any element of the resting nucleus. It is possible that this dissidence rests upon technical differences in the employment of Feulgen's method, but it is not yet certain that this is so—despite the plausible criticisms of several writers.

The only worker—so far as I know—who has previously studied *E. nana* by the Feulgen technique is Lévitanskaïa (1938). She obtained a positive reaction (indicating 'chromatin') with the granules in the nuclear membrane and sometimes with the karyosome, and her rough figures apparently show staining effects but little different from those obtainable with haematoxylin or other 'basic' dyes. This is wholly contradictory of my own experience: yet here again it is possible that the discrepancies are due to the different staining methods employed. Indeed, Lévitanskaïa—who worked with faecal films and not with cultures—says that she was unable to obtain any positive reaction by the usual technique, and succeeded only when she prolonged the time of hydrolysis to 16 minutes (with $N/_1$ HCl at 60° C.) and greatly increased the acidity of the fuchsine solution. Neither of these procedures has produced positive results in my hands.‖

* Cf. Chatton (1910), Dobell (1914), etc.

† Cf. Bělař (1926), etc.

‡ Cf. Jírovec (1927), Bogdanowicz (1930), etc.

§ Cf. Wermel (1925), Reichenow (1928), Pietschmann (1929), etc.

‖ Lévitanskaïa's results with *Dientamoeba*, however, appear to agree closely with my own. I regret that when I wrote my paper (Dobell, 1940) on this organism I was unaware of her work, which was briefly recorded in a Russian journal then inaccessible.

I shall not attempt to define 'chromatin', or to adjudicate on the question whether haematoxylin, Giemsa's stain, or Feulgen's reagent affords the truest test for its identification. Chromosomes are definite structures, and can be recognized by their morphology and behaviour at nuclear division. They can be stained by various methods, and accurately studied as solid bodies—no matter whether they be artificially coloured black, red, or purple. I have attempted no more in the case of *E. nana*, and must leave the more abstruse theoretical and chemical interpretation of my findings to others.

Assuming that my description of the polymorphism of the karyosome in *E. nana* is correct, and not a mere record of fixation artifacts or degenerative changes, I will add a further word about the interpretation of the phenomenon. I have already noted that the transformations which this body undergoes in the trophic amoeba appear to be irregular and variable, and I have concluded that they bear no relation to the phases of mitosis. That this inference is correct is supported by evidence furnished by other stages in the life-history. In the first place, I may note that the mitotic divisions which occur in the nuclei within the cysts are closely similar to those seen in trophic amoebae—though they are, of course, much smaller. All phases appear alike: yet in the cysts the nuclei, when at rest, do not exhibit the extreme karyosomic polymorphism so characteristic of trophic organisms. Secondly, during the metacystic stages no mitotic divisions at all occur: yet the nuclei—at all 'resting' stages—again show the same peculiar and variable forms of karyosome. Since these structural modifications may occur, therefore, in amoebae whose nuclei divide later by mitosis, and in others which do not so divide; and as they do not precede mitosis at certain other stages—the prophases and other phases being otherwise identical—it appears highly improbable that karyosomic polymorphism is in any way connected with mitotic division.

I emphasize this especially—for it must be sufficiently clear already from my preceding descriptions—because Brumpt & Lavier (1935*a*) have recently described a 'new genus' of intestinal amoebae (*Hyalolimax*) from an African monkey (*Cercopithecus ruber*): and though their account is confessedly incomplete and obviously faulty, it reveals clearly enough that they were really dealing with an *Endolimax*—closely similar to, and possibly identical with, *E. nana*. They observed the polymorphism of the karyosome in *Hyalolimax*, but failed to realize that it is characteristic of *Endolimax*; and they regarded certain common forms of the variable karyosome as prophases of mitotic division. (Other stages of mitosis described and figured seem not essentially different—when allowance is made for the paucity of material studied, and imperfections of technique—from those observable in *E. nana*.) The cysts of '*Hyalolimax*' have not been described, but I expect that when they are discovered they will prove to be closely similar to those of *E. nana*. In short, I have little doubt that *Hyalolimax* is merely another synonym of *Endolimax*.

Apart from these fragmentary observations on the division of '*Hyalolimax*', no account has previously been published of mitosis in any species of *Endolimax*.* I do not intend now to compare my findings with those of other workers on other amoebae, but I would emphasize the chief points of interest which have emerged from my own work—the intranuclear spindle, with large intranuclear centrioles and well-developed centrodesmus; the granular chromosomes (10), and their annular arrangement on the equatorial plate;† the persistence of the nuclear membrane during all early stages; and the characteristic telophases. These, together, form adequate features to characterize the genus.

Only one previous author—so far as I am aware—has described the process of excystation in *E. nana*. This is Hegner (1927), whose casual observations are hard to understand. He says—wishfully but quite wrongly—that 'moisture and a temperature of about 37° C. for several hours are the only factors necessary to stimulate excystation in the intestinal protozoa of man': and accordingly he claims to have witnessed the hatching of faecal cysts of *E. coli*, *E. histolytica*, and *E. nana* 'in water or in weak saline solution' kept at body-temperature. In my experience none of these species will undergo excystation under such conditions, and the added details only make matters more perplexing. *E. histolytica* is described as excysting in a manner similar to that really characteristic of *E. coli*,‡ while *E. coli* is then said to excyst like *E. histolytica*.§ 'Excystation could not be studied as easily in *Endolimax nana* as in *Endamoeba coli* because of its minute size. So far as could be observed, however, the process was similar in every respect.' In other words, the process is similar to what really happens in *E. histolytica*—not *E. coli*—which is contradictory but (unintentionally) nearly correct. It now appears singular that Hegner never published any fuller account of these researches, which leave so much to be explained. It is also singular that, in his review of earlier work on the same subject, he made

* I leave out of account the sketchy and uncertain notes on the division of *Endolimax blattae* published by Lucas (1927): and I have already indicated elsewhere (Dobell, 1940, p. 449) that Kudo's description (1926) of division in *Dientamoeba*—studied in a mixed infection with *E. nana*—may possibly be partly based on the latter organism.

† Cf. Dobell (1914).

‡ See Dobell (1938). § See Dobell (1928).

no mention of the fact that I had already hatched the cysts of all these species by different methods and had recorded this in the previous year.*

The metacystic stages of *E. nana* have not previously been studied or described. They differ notably from those of *E. histolytica*† and *E. coli*,‡ being in general far simpler. They bear a resemblance, however, to corresponding stages in *Entamoeba terrapinae* as described by Sanders & Cleveland (1930), who state that in this species the 4-nucleate metacystic amoeba—derived from the cyst—first undergoes simple fission, without nuclear divisions, into 2 binucleates, and each of these then divides into 2 uninucleate amoebulae. But the evidence presented for these statements, as I have pointed out elsewhere§, is by no means convincing.

Of the 5 species‖ of amoeba which live in the human intestine, I have now worked out the complete life-histories of 4¶—as far as this has been possible under the limitations of technical difficulties and contemporary circumstances. With the exception of *Dientamoeba*—which I now have reason to regard as not a true amoeba but a flagellate**—the life-histories of all have proved to be similar, though they vary greatly in detail. The trophic amoebae of all species multiply by simple binary fission, with mitotic division of the nucleus, and in no other way: and every species (except *Dientamoeba*) forms a characteristic multinucleate cyst—the final resting and infective stage—which is capable of prolonged survival at temperatures below that of the human body. When the cyst hatches in the culture-tube—and presumably in the human intestine likewise—it liberates its entire contents as a single multinucleate metacystic amoeba, which afterwards divides into a definite number of little amoebulae that grow into adult trophic forms once more. The details of development are different in each species, but the ultimate result is the same. The life-history, in general, is thus simple and asexual. There are no sexual stages—no conjugation or gametes during metacystic development, and no 'autogamy' in the cysts. There is nothing, indeed, to support the imaginative accounts of amoebic life-cycles published 30–40 years ago by German workers.

Accordingly, it can now be stated with some confidence that the wonderful descriptions of Schaudinn and of all his many imitators—which have exercised so profound an influence for so many years—were not founded on fact. They were, actually, so utterly wrong that even discussion of this early work—almost universally accepted when I began my researches—is now no longer necessary.

SUMMARY

1. This memoir amplifies my accounts of *Endolimax nana* published in 1919 and 1933. The complete life-history, as observable in cultures, is now described—special attention being devoted to the nuclear structure at all stages.

2. Additional information is also given about methods of cultivation, viability of the cysts *in vitro*, the 'nuclear reaction', natural parasites (*Sphaerita*, etc.), and other details.

3. The life-history has been found to comprise trophic, precystic, cystic, and metacystic stages, comparable with those described in *Entamoeba histolytica* (1928) and *E. coli* (1938).

4. The trophic amoeba is redescribed, with special reference to the polymorphism of its karyosome.

5. Division of the trophic amoeba (by binary fission) has been studied, and the mitosis of its nucleus is now first described in detail. It is characterized by the formation of an intranuclear spindle, with well-developed centrioles and centrodesmus. The granular chromosomes are 10 in number, and arranged in a ring on the equatorial plate.

6. Precystic amoebae are smaller than trophic forms—the reduction in size being effected during the last two divisions before encystation.

7. The cyst is formed by a precystic amoeba in the usual way. Inside the cyst, the nucleus undergoes two successive mitotic divisions similar to those seen in trophic forms. The ripe cyst is therefore 4-nucleate, though supernucleate specimens—which are viable—occur occasionally. Glycogen is normally present in young (1- and 2-nucleate) cysts, but not in those which are mature.

8. The process of excystation is described for the first time, and closely resembles that seen in *E. histolytica*. The entire protoplasmic contents emerge as a single 4-nucleate amoeba through a minute pore in the cyst wall.

9. Metacystic development—not previously studied—consists in simple division, by successive cytoplasmic bipartitions without division of the nuclei, into 4 uninucleate amoebulae. These grow into trophic amoebae once more.

10. The whole life-history is thus asexual, with no gametes, conjugation, or autogamy, at any stage.

* See Dobell & Laidlaw (1926), and Dobell (1927). I do not think we need now ascribe more importance to the late Dr Hegner's words than he appears to have attached to them himself.

† Cf. Dobell (1928). ‡ Cf. Dobell (1938).

§ Dobell (1938), pp. 228–9.

‖ I showed that there were probably only 5 species in 1919, and all subsequent researches—and mistakes—have confirmed this conclusion.

¶ The fifth—*Iodamoeba*—is still under investigation, and I hope to describe its life-history later.

** Cf. Dobell (1940).

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EXPLANATION OF PLATES I AND II

All figures depict fixed and stained specimens of *Endolimax nana*. Magnification 2000 diameters (2 mm. = 1 μ) throughout. All drawings made, with aid of *camera lucida*, under Leitz 2 mm. apochromatic oil-immersion objective (N.A. = 1·40) with compensating oculars and achromatic aplanatic condenser.

The description of each figure is followed by a number [in square brackets] which is my serial number attached to the permanent preparation in which the specimen illustrated is to be found. Particulars of each slide are given below—the following abbreviations being used for fixatives and stains:

SAA. = Schaudinn's sublimate-alcohol + 5% glacial acetic acid.
Bo. = Bouin's fixative (original aqueous formula).
FeHx = Heidenhain's iron-alum haematoxylin.
PWHx = Mallory's phosphotungstic-acid haematoxylin.
FeClC = Hollande's iron-chlorocarmine.

A number in parentheses following the designation of the strain—e.g. *LO.* (42)—indicates the serial number of the subculture. For culture-media the abbreviations are those used by Dobell & Laidlaw (1926).* A time (in hours) denotes the period for which the culture was incubated at 37° C. before the specimen was fixed.

1799 = Strain *LO.* (7) Ehs. 48 hours. SAA. FeClC.
1810 = Strain *LO.* (42) HSre + broth. 6½ hours. SAA. PWHx.
1812, 1813 = Strain *LO.* (48) Ehs. 48 hours. SAA. PWHx.

* Cf. p. 135 *supra*.

1849, 1850 = Strain *LO.* (58) HSre + meat extract. 6½ hours. SAA. PWHx.
1851, 1852 = Strain *LO.* (58) HSre + meat extract. 24 hours. Bo. PWHx.
1996 = Strain ΔX. (27) Ehs. 2½ hours. SAA. FeHx.
2081 = Strain *dx.* (14) Ehs. 2½ hours. SAA. FeHx and erythrosin.
2234 = Strain *SD.* (101) Ehs. 5½ hours. SAA. PWHx.

Figs. 1–21 are from preparations fixed by the coverglass technique described elsewhere (Dobell, 1942): the rest are from ordinary smears.

PLATE I

Figs. 1, 2. Active trophic amoebae, showing two common forms of nucleus. [2234]

Figs. 3–21. Stages in division of trophic amoebae

Figs. 3, 4. Early prophases. [1996]
Figs. 5, 6. Nuclei only: prophases. [2234]
Figs. 7, 8, 9. Prophase stages. [1996]
Fig. 10. Equatorial plate and intranuclear spindle. [1996]
Fig. 11. The same. [2234]
Fig. 12. Nucleus only: metaphase. [2234]
Fig. 13. Nucleus only: early anaphase. [2234]
Fig. 14. Early anaphase: whole organism. [1996]
Fig. 15. Anaphase. [1996]
Fig. 16. Late anaphase. [2081]
Fig. 17. Early telophase. [1996]
Figs. 18–21. Final telophases: successive stages in formation of daughter-amoebae. [1996]

Figs. 22–25. Precystic fission

Fig. 22. Prophase. [1813]
Fig. 23. Equatorial plate stage. [1812]
Fig. 24. Telophase. [1813]
Fig. 25. Late telophase: separation of daughter-amoebae. [1813]

Figs. 26–30. Precystic amoebae

Fig. 26. Small form, with central karyosome. [1813]
Figs. 27, 28. Larger specimens, with peripheral karyosomes. [1813]
Fig. 29. Very small form, with central karyosome. [1799]
Fig. 30. Large specimen, with central karyosome, glycogen vacuole, and volutin grains. [1812]
Fig. 31. Encystation. [1813]

Figs. 32–41. Successive stages in development of cysts

Fig. 32. Uninucleate cyst. [1812]
Fig. 33. Prophase, first nuclear division in cyst. [1813]
Fig. 34. Spindle stage, first nuclear division. [1813]
Fig. 35. Anaphase, first nuclear division. [1813]
Fig. 36. Binucleate cyst. [1813]
Fig. 37. Prophase, second nuclear division in cyst. [1813]
Figs. 38, 39. Equatorial plate stages, second cystic division. [1813]
Fig. 40. Final stages of second division: one nucleus divided, the other in late telophase. [1813]
Fig. 41. Mature quadrinucleate cyst. [1812]
Fig. 42. Supernucleate (5-nucleate) cyst. [1799]
Fig. 43. Supernucleate (8-nucleate) cyst. [1799]
Fig. 44. Very small uninucleate cyst. [1812]
Fig. 45. Very small ripe (4-nucleate) cyst. [1812]
Fig. 46. Mature 4-nucleate cyst with bacterial (?) inclusions. [1799]
Fig. 47. Cyst with filamentar (bacterial?) inclusion. [1810]

Figs. 48, 49. Examples of cannibalism

Fig. 48. Precystic amoeba ingested by a trophic amoeba. [1813]
Fig. 49. Phagocytosis of 4-nucleate cyst by a trophic amoeba. [1799]

PLATE II

Figs. 50–62. Excystation
Figs. 63–102. Metacystic stages

Fig. 50. Ripe 4-nucleate cyst. [1849]
Fig. 51. Clarification of cytoplasm on incubation. [1850]
Fig. 52. Separation of protoplasm from cyst wall. [1849]
Fig. 53. 4-nucleate amoeba loose in cyst. [1849]
Fig. 54. Amoeba penetrating cyst wall. [1850]
Figs. 55–59. Successive stages in emergence of amoeba [1849]
Fig. 60. Newly hatched amoeba sitting on empty cyst. Nuclei clumped. [1850]
Fig. 61. Hatchling moving away from empty cyst. [1850]
Fig. 62. Very small amoeba, just hatched. [1850]
Fig. 63. 4-nucleate metacystic amoeba. [1852]
Fig. 64. Ditto. [1849]
Fig. 65. Ditto, larger specimen. [1851]
Fig. 66. Ditto, small specimen. [1849]
Fig. 67. 4-nucleate metacystic amoebae in which all nuclei show peripheral distribution of chromatin. [1850]
Fig. 68. Supernucleate (5-nucleate) cyst, preparing to hatch. Amoeba loose in cyst. (Cf. fig. 53.) [1849]
Fig. 69. Later stage of same: 5-nucleate amoeba just hatched from cyst. [1849]
Fig. 70. Large 5-nucleate metacystic amoeba. [1851]

Figs. 71–74. Division of 4-nucleate amoeba into a 3-nucleate and a 1-nucleate

Fig. 71. Separation of 1 nucleus from other 3. [1810]
Fig. 72. Fission of cytoplasm. [1851]
Figs. 73, 74. Trinucleate and uninucleate products of such fission. [1851]
Fig. 75. Larger 3-nucleate amoeba. [1850]

Figs. 76–79. Division of 3-nucleate amoeba into a 2-nucleate and a 1-nucleate

Fig. 76. Separation of nuclei: beginning of fission. [1852]
Fig. 77. Final stage of fission into 2-nucleate and 1-nucleate. [1851]
Figs. 78, 79. Products of such fission—small binucleate [1851] and uninucleate amoebula [1810].
Fig. 80. Large 2-nucleate metacystic amoeba, with central karyosomes. [1851]
Figs. 81–84. Large binucleates, showing various other nuclear arrangements. [1851]

Figs. 85, 86. Division of 4-nucleate amoeba into 2 binucleates

Fig. 85. Separation of nuclei: cytoplasmic fission beginning. [1851]
Fig. 86. Final stage of fission: larger specimen. [1851]
Fig. 87. Small 2-nucleate amoeba. [1851]

Figs. 88–90. Division of binucleate amoeba into 2 uninucleate amoebulae

Fig. 88. Separation of nuclei. [1851]
Fig. 89. Beginning of cytoplasmic fission. [1852]
Fig. 90. Cytoplasmic fission almost complete. [1851]
Fig. 91. Very small uninucleate amoebula. [1851]
Fig. 92. Large uninucleate, with karyosome still central. [1851]
Figs. 93–97. Uninucleate amoebulae, showing various nuclear arrangements. [1851]
Fig. 98. Young trophic amoeba. [1851]
Fig. 99. Larger specimen. [1852]
Fig. 100. Very large 3-nucleate metacystic amoeba, with peripherally distributed chromatin. [1851]
Fig. 101. Very large 4-nucleate, with similar nuclei. [1852]
Fig. 102. Supernucleate (6-nucleate) metacystic amoeba dividing into 2 trinucleate products. [1851]

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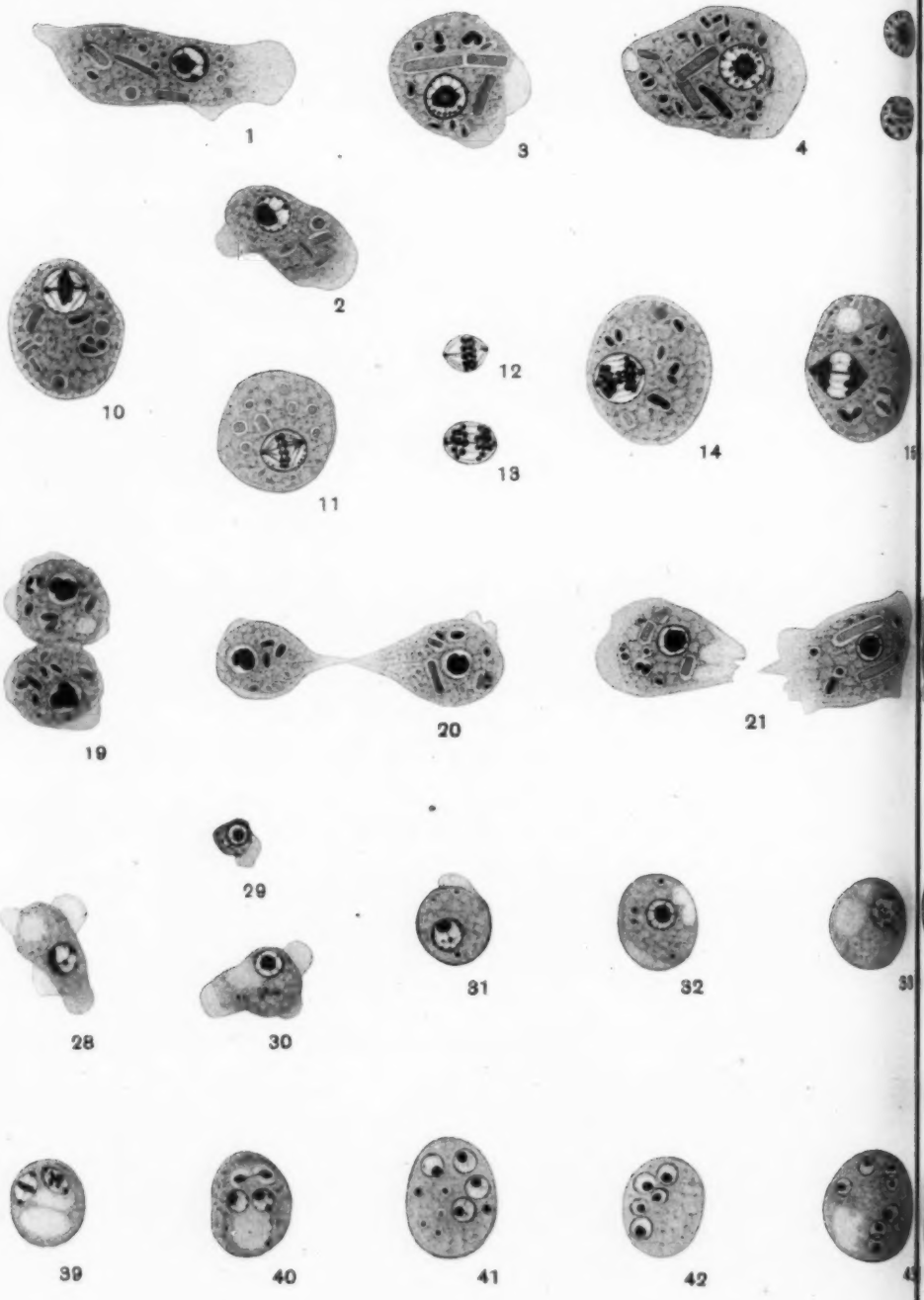

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PLATE I

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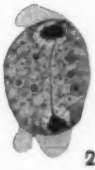

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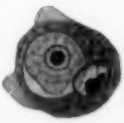

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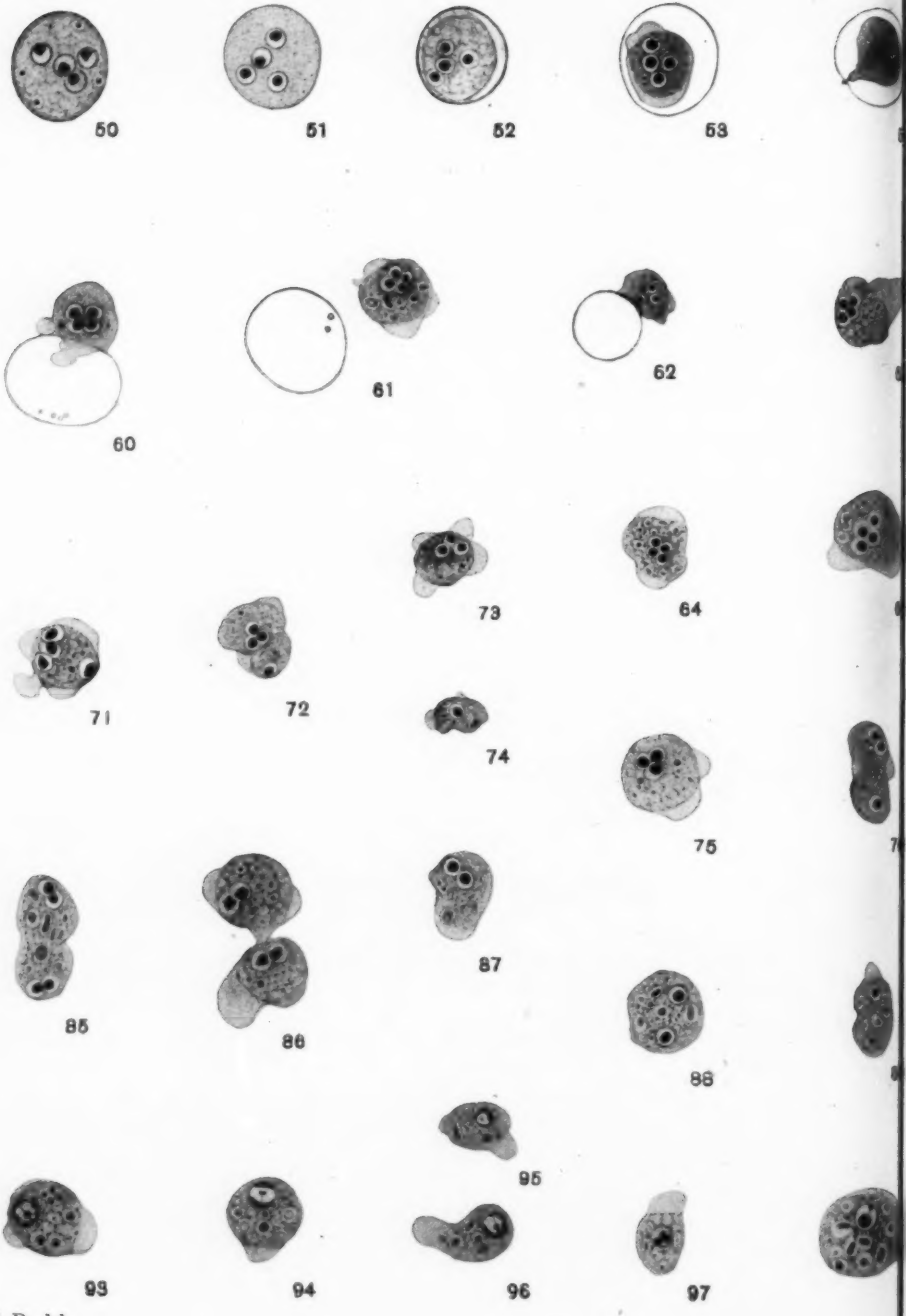

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PLATE II

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56
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92
88
84
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100
101
102

A VIRUS DISEASE OF *ATROPA BELLADONNA*

By KENNETH M. SMITH, F.R.S., *Plant Virus Research Station and Molteno Institute, Cambridge*

(With Plate III)

INTRODUCTORY

During the summer of 1941 some specimens of *Atropa belladonna* were received from Suffolk, apparently affected with a virus disease. Experiment showed that a virus was in fact concerned, and, moreover, suggested that the virus was one not so far described. Further work has now confirmed this, and since the disease caused to an economic crop is a serious one, it was considered worth while publishing a preliminary account of it. During 1942 further cases of the disease have been recorded in Surrey, and where *A. belladonna* is being grown for medicinal purposes the virus seems to be of some importance. So far as the writer is aware this is the first time that a naturally occurring virus disease has been recorded affecting *A. belladonna*.

THE VIRUS

Transmission. The virus is sap-inoculable and is fairly infectious. Insect-transmission experiments have not been completed, but preliminary tests suggest that neither the aphis *Myzus persicae* nor *Thrips tabaci* are the vectors.

Dilution end-point. In crude extracted sap the dilution end-point lies between 1 : 10,000 and 1 : 1000.

Thermal inactivation point. The virus appears to be relatively resistant to heat and is not destroyed by 5 min. exposure to 75° C. The inactivation point probably lies between 75 and 80° C.

Longevity in vitro. Positive infections were obtained after 6 days' ageing in extracted sap at room temperature but not after 11 days.

Symptomatology and host range

Atropa belladonna. Affected specimens as received from the outdoor crop showed severe necrosis of the leaves and stem. Under glasshouse conditions the symptoms consist of a rather patchy mottle on the leaves together with numbers of necrotic spots. Much difficulty has been experienced in infecting *belladonna* with the virus by mechanical means.

Hyoscyamus niger. This plant seems to be quite susceptible to infection. On young plants local lesions are formed and all plants later show a systemic mosaic mottle which is rather pale and never very pronounced. There is less necrosis than with many other hosts.

Solanum nigrum. This plant is susceptible to infection but the symptoms are faint and indeterminate. A very slight dark green mottle together with a small amount of rugosity of the leaf surface are the only symptoms. The virus, however, can be recovered from such plants by inoculation tests to *Nicotiana glutinosa*. *Solanum nigrum* may play some part in harbouring and spreading the virus.

Datura Stramonium reacts to infection with a faint mosaic mottle, there is no necrosis;

Nicotiana tabacum var. White Burley. The tobacco plant is very susceptible to infection by inoculation, and the symptoms are entirely necrotic. Local lesions develop after about 5 days and may consist of ring-like or solid necrotic spots. There is a strong tendency for the necrosis to spread to the midribs and larger veins and thence to the stem. Frequently necrosis develops low down in the main stem causing wilting and death of the whole plant. Mosaic mottling has not been observed in tobacco with this virus.

Nicotiana tabacum var. Turkish. The symptoms on this variety are rather similar to those on White Burley, but systemic necrosis in the form of ring and line patterns seems to be rather more pronounced than in White Burley. Necrosis of the stem and midribs is also common.

Nicotiana glutinosa. The reaction of this plant to the virus is entirely necrotic and the plant is almost invariably killed. Local lesions are produced on the inoculated leaves which in their early stages closely resemble those produced by tobacco mosaic virus on the same host (Pl. III, fig. 2). Later, however, the spots enlarge and spread through the leaf, sometimes giving rise to single incomplete rings. Local symptoms are followed by systemic necrosis of the stem which usually kills the plant.

Phaseolus vulgaris French Bean var. Canadian Wonder. The virus gives rise to local lesions on French bean which are discrete and easy to count. They do not spread in the manner of the lesions of tobacco necrosis on the same plant. There is no systemic infection of French bean. The local lesions resemble rather closely those produced by tobacco mosaic virus on the variety of French bean Early Golden Cluster.

DISCUSSION

From its properties and symptomatology there is little doubt that the *belladonna* virus has not been previously described. Whether it will prove to be of serious economic importance on herb farms and other places where *belladonna* is grown or whether it will disappear again like the virus of tomato bushy stunt, one cannot say. More work needs still to be done on the question of insect transmission of the virus, and the insect fauna of *belladonna* must be examined from this viewpoint. So far as the preliminary tests have gone, however, it seems as if this virus was not insect-borne.

The virus itself will probably be suitable for further study, since it is stable with a high thermal inactivation point, occurs in high concentration in the plant, and gives good local lesions on *Nicotiana glutinosa* and *Phaseolus vulgaris*.

SUMMARY

A virus disease of *Atropa belladonna* caused by a new virus is described. The virus is sap-transmissible to tobacco and other solanaceous plants and gives good local lesions on *Nicotiana glutinosa* and *Phaseolus vulgaris*. It occurs in fairly high concentration in the plant, it withstands ageing in extracted sap for 6–11 days, and is inactivated at a temperature between 75 and 80° C.

EXPLANATION OF PLATE III

Fig. 1. Plant of *Atropa belladonna*, natural infection, showing mottling and slight necrosis of the leaves.

Fig. 2. Leaf of *Nicotiana glutinosa*, showing the numerous local lesions produced by the virus.

Fig. 3. Leaf of *Nicotiana glutinosa* showing extensive necrosis, younger plant than in fig. 2.

Fig. 4. Leaf of *Nicotiana glutinosa* showing the kind of necrosis produced by systemic infection.

(Photographs by Roy Markham)

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Fig. 1

Fig. 2

Fig. 3

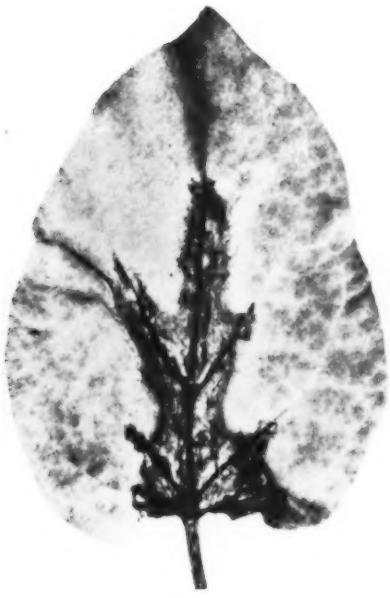

Fig. 4

THE VIRUS

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CONTENTS

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PAGE

BALDWIN, ERNEST. An *in vitro* method for the chemotherapeutic investigation of anthelminthic potency. (With 11 Figures in the Text) . . 89

BAYLIS, H. A. Some nematode parasites of earthworms from the Indo-Malay region. (With 38 Figures in the Text) 112

KEEGAN, HUGH L. Three new ophidian lung mites of the subfamily Entonyssinae Ewing, 1922. (With 14 Figures in the Text) . . . 128

DOBELL, CLIFFORD. Researches on the intestinal protozoa of monkeys and man. XI. The cytology and life-history of *Endolimax nana*. (With Plates I & II and 1 Text-figure) 134

SMITH, KENNETH M. A virus disease of *Atropa belladonna*. (With Plate III) 159

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